CONSTITUTIVE MUTATIONS IN THE PENICILLINASE SYSTEM OF STAPHYLOCOCCUS AUREUS

A contribution towards an understanding of the control mechanisms of enzyme biosynthesis

by

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I wish to dedicate this thesis to my sister whose personal example and cheerful encouragement was a continual help to myself as it was also to everyone who knew her.

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SUMMARY

Mutants of S. aureus, constitutive for penicillinase synthesis, with the phenotypes magno-constitutive, meso-constitutive, and micro-constitutive were examined. Complementation experiments have shown that the inducible regulation of penicillinase synthesis is determined by two cistrons. The cistron i_R determines the repressed level of enzyme activity and the cistron i_N the level of enzyme synthesised in the presence of inducer. In a magno-constitutive mutant it has been shown that only i_R is mutated, and in a micro-constitutive mutant both i_N and the structural gene for the penicillinase protein (p) were mutated. In a meso-constitutive mutant both i_R and i_N were mutated, and the mutational events were not resolvable by recombination. No evidence for an operator region was found, a search for operator-constitutive mutants being completely negative. The control of penicillinase synthesis in S. aureus is discussed with respect to other constitutive mutants, not examined here, and to other bacterial control systems.

Contents

Experimental Results

I. INTRODUCTION

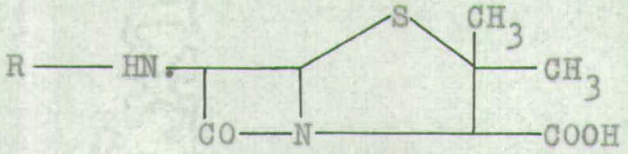
Staphylococcus aureus is a Gram-positive coccus, which is potentially pathogenic and normally sensitive to benzylpenicillin (Fig. 1), an antibiotic that interferes with the synthesis of the bacterial cell wall (Rogers, 1967). Strains of S. aureus isolated from clinical infections are frequently penicillin-resistant, and all such isolates destroy penicillin (Barber, 1962) due to the production of an enzyme, penicillinase (EC 3.5.2.6), which hydrolyses the amide bond in the β-lactam ring of the penicillin "nucleus" (6-APA) to produce penicilloic acid (Fig. 2), a molecule devoid of antibacterial activity. As the enzyme also hydrolyses other penicillins, and, less actively, a similar bond in the related group of compounds, the cephalosporins (Fig. 1), the general name of β-lactamase has been proposed for enzymes of this type by Citri & Pollock (1966) in their extensive review of these enzymes.

The rate at which the different penicillins and cephalosporins are hydrolysed and the affinity for the substrate (K_m) vary with the source of the enzyme and the substrate used. That the range of substrate activity is the property of a single, broad spectrum enzyme in each case was shown by Richmond (1963) with purified and mutant enzyme preparations.

No accessibility barrier between the enzyme and the substrate was found by Geronimus & Cohen (1958), Batchelor, Cameron-Wood, Chain & Robinson, (1963), and Richmond (1965a), but Depue, Moat & Bondi (1964) obtained an increase in enzymic activity after

Fig. 1. The structure of penicillins and related compounds.

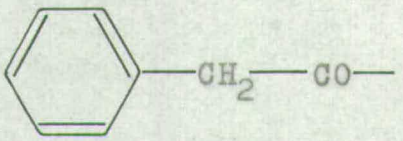
(a) General structure of penicillins, N-acyl derivatives of 6-aminopenicillianic acid (6-APA).



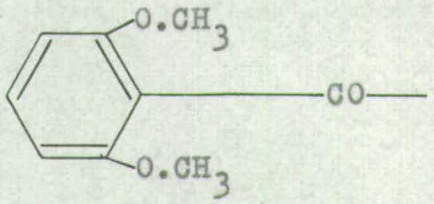
(b) R-groups

(i) 6-APA R = H—

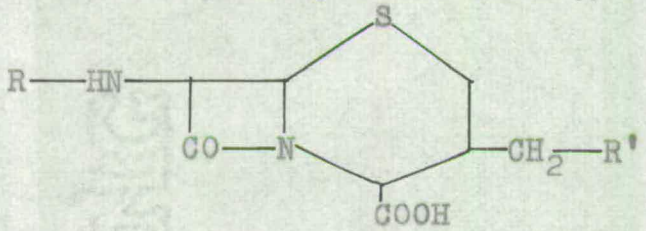
(ii) benzylpenicillin R =



(iii) methicillin R =

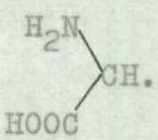


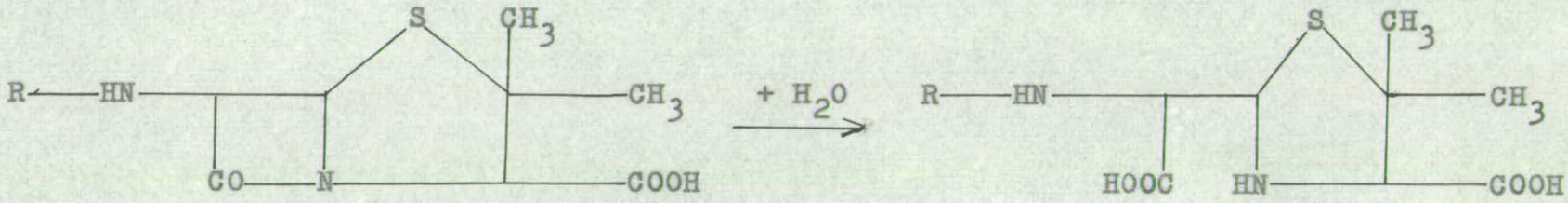
(c) General structure of cephalosporins, N-acyl derivatives of 7-aminocephalosporanic acid (7-ACA)



(d) R-groups and R' groups

(i) 7-ACA R = H— R' = $-OOC.CH_3$

(ii) Cephalosporin C R =  $CH.(CH_2)_3.CO-$ R' = $-OOC.CH_3$



penicillin

penicilloic acid

Fig. 2. The reaction catalysed by penicillinase.

disrupting the bacterial cells. This need not necessarily mean release of cryptic enzyme, but may be due to a change in the enzyme on solubilisation. Some differences between the cell-bound and solubilised penicillinase of their strain of staphylococcus were noted by Batchelor and his colleagues.

Three varieties of naturally occurring staphylococcal penicillinase (A, B, C) have been identified by Richmond (1965a) on immunological and enzymological grounds. Type B seems to be confined to Staphylococci of phage group II, while A and C are found in groups I and III. Minor differences in the amino-acid composition of A and C were also reported. The work reported in this thesis is concerned only with types A and C.

Staphylococcal penicillinase is an extracellular enzyme (Gots, 1945), being released from the cells into the culture medium, the degree to which this occurs being genetically determined for each strain, and varying also with the medium and phase of growth (Novick, 1962a; Swallow & Sneath, 1962; Novick & Richmond, 1965; Citri & Pollock, 1966; Coles & Gross, 1967 a & b). The enzymic properties of the exoenzyme are essentially the same as those of the cell-bound enzyme (Richmond, 1963, 1965 b) sometimes with slight differences in the substrate affinity (Novick 1962a) or in inactivation by the substrate (Dyke, 1967), thought to be due to removal of molecular constraints. For all three enzyme types, the exopenicillinase is a single protein unit of molecular weight about 30,000, containing neither cysteine nor tryptophan (Ambler; cited by Citri & Pollock, 1966).

Synthesis of penicillinase is inducible in naturally occurring strains (Bellamy & Klimek, 1948; Geronimus & Cohen, 1957, 1958). All the penicillins tested, the penicillin "nucleus", 6-APA, and a number of cephalosporins have been shown to be inducers for the enzyme (Crompton, Jago, Crawford, Newton & Abraham, 1962; Knox & Smith, 1962; Swallow & Sneath, 1962). Effective induction requires the continued presence of the inducer molecule, so that it is difficult to obtain the conditions for maximal induction with compounds which are rapidly hydrolysed by the enzyme, for example, with benzylpenicillin, phenoxymethyl penicillin, phenethicillin, and phenoxypropyl penicillin, or which are insoluble, as, for example, with benzylpenicillin amide (Batchelor et al., 1963; Knox & Smith, 1962; Swallow & Sneath, 1962; Novick, 1962a). The maximum amount and efficiency of induction obtained with compounds which are not rapidly hydrolysed (gratuitous inducers) varies with the structure of the compound and may be masked by the intervention of antibacterial action at higher concentrations. A number of compounds which are gratuitous inducers hydrolysed at 15% or less of the rate for benzylpenicillin, are shown in Table 1, together with the minimal concentration inhibiting bacterial growth, and the "induction constant" (Pollock, 1957), defined as the concentration necessary to induce enzyme formation 50% of the maximal rate, and an indication of the maximum amount of enzyme formed under the conditions used by the authors.

The amount of enzyme made, both with and without induction, and the rate of induced enzyme synthesis differ with the composition

Table 1. The effect of different penicillins and cephalosporins on S. aureus: The properties as substrates and inducers of penicillinase and the antibacterial potency.*

Compound	Rate of hydrolysis by penicillinase relative to benzylpenicillin (100)	Induction		Minimum growth inhibitory concentrations (μg./ml.) with resistant staphylococcus
		Apparent induction constant≠ (μmg./ml.)	Ratio of maximum induced penicillinase activity to uninduced activity	
Benzylpenicillin	100	10->30	11	>1000-<0·9
Methicillin	0·3-5	0·3	40-65	7·5 - 0·2
Picramidopenicillanic acid	11	<5	50-100	>200 - 6·2
Oxacillin	0·8-7	0·02	60	
6-APA	1-19	3·0	20-60	500 - 50
Cephalosporin C	<0·1-1·2	10	30-50	125 - 100
Deacetylcephalosporin C	0·1	13		500
Cephalosporin C_a (pyridine)	1·2	0·9		8
Cephalosporin C_c	15 ± 5	>50		900
N-Phenacetyl-7-ACA	<0·1	0·01		0·5
Reference≠	(2)(3)(6)	(3)(4)(6)	(2)(5)(6)	(1)(3)(4)(6)

* The purpose of this Table is to give a comparison of the properties of different penicillins and cephalosporins, and it is compiled from several different authorities. Each give their data in an individual form so that strict comparison is not possible and the absolute values of some entries may be unreliable.

≠ See text.

≠ (1) Batchelor et al. (1963); (2) Citri & Pollock (1966); (3) Crompton et al. (1962); (4) Knox & Smith (1962); (5) Novick (1962a); (6) Swallow & Sneath (1962).

of the medium, such as amino acid and hexose content, and with some cations, particularly at low pH values. This effect is slight at neutral pH (as used for the work described in this thesis) and differs with the size and age of the inoculum (Batchelor et al., 1963; Cohen, Sweeney & Leitner, 1967; Geronimus & Cohen, 1957; Kaminski, 1962; Kaminski, Bondi, de St. Phalle & Moat, 1959; Knox & Smith, 1962; Leitner, 1967; Leitner & Cohen, 1962; Leitner, Sweeney, Martin & Cohen, 1963; Steinman, 1961). The differences are probably due in part to the fact that induced penicillinase synthesis requires bacterial growth and the rate of enzyme synthesis is proportional to the growth rate (Batchelor et al., 1963). A variety of times required for maximum induction have been reported by the different authors. There is a characteristic lag period before expression of induced penicillinase, the increase being detectable at about 15 min. with methicillin induction, but the maximum rate of synthesis does not appear till over 40 min. (Batchelor et al., 1963; Novick, 1962a; Steinman, 1961). Novick (1962a), using conditions similar to those used for the work described below, found a lag phase of about 50 min. followed by a logarithmic rate of enzyme production during the log phase of growth. The total enzyme reached a maximum at the end of the log phase (about 6 hr.) and declined during the stationary phase. Exoenzyme activity was greatest at about 3 hr.

Two closely linked genetic regions governing penicillinase production were originally identified by Novick & Richmond (1965). These are the structural gene (p), determining the polypeptide

sequence of the enzyme, and a regulator gene (*i*), which confers inducible properties on enzyme synthesis.

Mutants of the structural gene for A-type penicillinase (p_A), producing immunological cross-reacting protein (CRM) with lowered enzyme activity have an amino acid substitution in the primary structure of the exoenzyme (R.P. Ambler & M.H. Richmond, unpublished work). These mutants are, however, still inducible, retaining the same proportional increase of activity as that found in the unmutated strain (Richmond, 1963).

Mutants of the regulator gene (*i*) have been reported which lose their inducible properties without affecting the structural gene. Derepressed mutants (i^-) make constitutively large amounts of enzyme that is identical with normal induced penicillinase (Richmond, 1963). Richmond (1965b) has shown that inducible control of the *p*-gene of these mutants is restored in heterozygous diploids by the wild type (i^+) allele in the *trans* position. This suggests that the control system is similar to that for β-galactosidase in *E. coli* (Jacob & Monod, 1961) where the regulator gene (*i*) determines a cytoplasmic repressor which for the *lac* operon is, at least partially, a protein (Bourgeois, Cohn, & Orgel, 1965; Muller-Hill, 1966; Gilbert & Muller-Hill, 1966). This repressor largely inhibits enzyme synthesis in the absence of inducer, but is altered in its presence to allow structural gene activity. Similarly, for the λ phage of *E. coli* the repressor is a cytoplasmic protein molecule (Ptashne, 1967), but it is possible that in other systems a different mechanism might apply. One such mechanism is discussed

in Section III.2 below. The types of constitutive, or non-inducible, mutants found for staphylococcal penicillinase are discussed in more detail below.

Another control locus closely linked to, but distinct from, i and p was suggested by Richmond (1965c; 1966a & b) on the basis of micro-mutants in which the amount of enzyme synthesised is severely restricted. This restriction affects only the structural gene colinear with the mutation, and is unaltered by the presence of other forms of the allele trans in polygenomic strains. In the first paper, Richmond (1965c) proposed that these are either polar mutations or mutations of the operator-zero (O) type in a locus filling some of the criteria for an operator locus as originally described by Jacob & Monod (1961); in the later papers further evidence is presented for the second of these hypotheses. The operator gene (O) as originally defined for the β-galactosidase system determined both the site of action of the repressor molecule, made by the regulator gene (i), and the site of initiation of expression (transcription or translation) of the regulated gene or genes comprising the operon. The postulated effects of mutations in O were either to cause the repressor to fail to bind effectively, or to cause loss of operon expression, or both, and would affect the adjacent genes only and irrespective of the wild type allele (O^+) in the trans position (or cis-dominantly). Mutants meeting these criteria were isolated in lac and identified as operator-constitutive (O^c) and operator-zero (O^o) respectively (Jacob & Monod, 1961). For the lac operon the two functions of

regulation and of gene expression have subsequently been shown to belong to two independent, though probably adjacent, genetic loci, that for the second function being renamed the promotor (Beckwith, 1964b; Jacob, Ullman & Monod, 1964). This is in contrast to the penicillinase system of S. aureus where Richmond (1966a & b) concludes that the characteristics of the micro-constitutive mutants are not readily compatible with subdivision of the operator region.

Another form of regulation of the penicillinase of S. aureus has been postulated by Baldwin & Hastowo (1965). These authors found that the colonies obtained by transducing the penicillinase gene from a naturally occurring micro-constitutive strain into another host strain were all magno-inducible, and ascribed the original constitutive phenotype to a positional effect of the transducing prophage. Cohen & Sweeney (1967, 1968) independently reported magno-constitutive mutants in another strain of S. aureus which give inducible penicillinase transductants in an unmutated, penicillinase-negative recipient. They demonstrated dual regulatory loci in their strain, of which one was cotransducible with the structural gene, as was the i-gene discussed above, and a second unlinked locus. Mutation at one or both loci evoked constitutive penicillinase formation. Further genetic or functional data are not available for either of these cases and the possibility that these are due to generalised suppressor strains or mutations affecting the repressor but not the enzyme molecule has not yet been excluded.

The proportion of enzyme present in the extracellular form is a genetic characteristic closely linked to the structural gene for penicillinase, and not specifically influenced by the genetic background of the host, but influenced by the growth conditions. It is not yet clear whether the degree of extracellularity is a property of the enzyme molecule itself (controlled by the p-gene) or is determined by another gene close to p. In support of the former postulate is the finding that two distinct varieties of penicillinase made by a heterodiploid strain are released independently of one another, and in the same proportions observed from cells making only a single type; while in favour of the latter is the observation that a series of naturally occurring staphylococcal strains make serologically identical penicillinase but have different liberation patterns (Novick & Richmond, 1965; Richmond, 1965b & c). Structural gene mutants (CRM^+) retain the liberation characteristics of the parental strain from which they were derived.

Staphylococcal penicillinase genes are usually located on an extrachromosomal element, or plasmid (Novick, 1963), although Asheshov (1966) and Poston (1966) have reported strains with a chromosomal location for the penicillinase. No integration of a plasmid into the host chromosome has yet been observed (Novick, 1967a). In addition to the penicillinase markers, a number of other genetic characters may be carried on the plasmid. These include resistance to a variety of metal ions, with independent loci for resistance to mercury (Moore, 1960; Richmond & John, 1964), cadmium and zinc, arsenate and arsenite, and lead (Novick, 1967a; Novick & Roth, 1967); resistance to macrolide antibiotics such as

erythromycin (Hashimoto, Kono & Mitsuhashi, 1964); and a rare colonial morphology marker (Richmond, 1965c). The host cell is neutral with respect to the expression of these characters in the strains so far examined.

On the basis of penicillinase type (A, B, C), degree of exocellularity, and pattern of resistance markers, the plasmids have been divided into types designated by letters of the greek alphabet (Novick & Richmond, 1965; Dyke & Richmond, 1967). So far 10 different plasmid types have been described.

The genetic determinants for penicillinase are readily transducible into new host strains (Ritz & Baldwin, 1961), the entire plasmid linkage group being generally transduced intact (Novick & Richmond, 1965). By this means, Novick & Richmond (1965) constructed a variety of different plasmid heterogenotes, and found that these either persisted as stable diploids or segregated rapidly, sometimes with recombination between the plasmids. On the basis of their ability to survive in the diploid state plasmids can be assigned to two compatibility groups (I and II); each plasmid is incompatible with members of its own group but compatible with members of the other.

Maintenance of the plasmid as an independent replicating unit and its compatibility properties may be affected by host cell mutations (Richmond, 1967; Novick, 1967a & b). Spontaneous loss of a plasmid normally occurs at 10^{-3} to 10^{-4} per cell generation from both haploid and diploid bacteria; this rate is greatly increased in mutant host strains or for certain plasmid mutants

(Novick, 1967b). All the plasmid-linked markers are usually lost simultaneously. As in practice all the known plasmid elements are cotransduced in over 99% of cases, or are lost together from the host cell, any of the linked markers may be used to tag the whole plasmid or select its transductants.

The work for this thesis is restricted to strains carrying plasmids α, β, and γ, used by Novick & Richmond (1965). The α and γ plasmids specify A-type penicillinase, more than 25% of which is normally extracellular, and belong to compatibility group I. Their main distinction is that the γ plasmid carries the marker for erythromycin resistance. Plasmid β belongs to group II and makes C-penicillinase, of which less than 10% is released into the medium. Heterozygotes involving α and/or γ plasmids segregate about 95% by the first cell division, and may recombine in the process. Heterozygotes of either α or γ (or an α --- γ recombinant) form stable polyplasmid cells with β, and recombination between the two plasmids is a rare event. Both structural genes are expressed equally in such diploids but the total amount of induced enzyme protein synthesised is similar to that of the haploid parent cells (Richmond, 1965b). The extracellularity of each enzyme type is expressed independently with its own characteristics.

In studying the control of penicillinase synthesis in S. aureus, use is made of the genetic tools of mutation, recombination between plasmids of the same compatibility group, and tests of complementation of function (Fincham, 1962, 1966; Hayes, 1964; Schlesinger & Levinthal, 1965) in stably diploid strains containing a plasmid

from each compatibility group. The behaviour of such diploids can be used to infer the allelism of two mutations, to assign them to different genetic loci, to show the dominant recessive relationship of two alleles, and to demonstrate interallelic complementation.

The basic ideas of bacterial genetics and complementation in micro-organisms have been described by Fincham (1966) and Hayes (1964). If the gene is defined as the region of the chromosome which determines a single function at the biochemical level, mutations of a gene, called alleles, will affect this one function. However, mutations demonstrating the same apparent phenotype may not necessarily be in the same gene. For example, if the observed character is the result of several sequential biochemical steps, alteration of any of these may equally affect the end product. If two mutations are introduced into a diploid cell on opposite chromosomal strands, (the trans configuration), they can complement one another to produce wild phenotype if they involve different functional units of the chromosome, while the mutant phenotype will generally continue to be expressed when the same functional unit is affected (but see below, P.12). The chromosomal unit of function recognized by this test was called the cistron by Benzer (1957) and is considered to be the unit that determines a single polypeptide chain. Thus a diploid strain constructed from mutants of two separate cistrons makes normal molecules of both proteins, which together give wild type activity both quantitatively and qualitatively. All mutants of one cistron complement all mutants of another cistron, and the complementation groups correspond to the units obtained by

recombination mapping.

When two alleles produce dissimilar phenotypes, for example a wild type (+) and its mutant derivative (-), and a diploid strain carrying both of them phenotypically resembles one and not the other, the allele whose function is expressed in the phenotype is said to be dominant and the other recessive. The term recessive is strictly applicable only in the case of an allele determining a function whose expression is subject to suppression by the function of another allele of the same gene in the trans position. In the case of a gene, the functions of whose alleles are expressed independently in a heterodiploid cell, neither allele is recessive but both are cis-dominant. Mutations in genes of this type may also lead to a cis-dominant alteration in the expression of genes adjacent to the mutation.

Pairs of similarly altered mutants which map together in the same locus generally continue to express the altered phenotype, and this is taken as evidence of allelism. However, some pairs may form active enzyme and this phenomenon is called intracistronic (or interallelic) complementation. The properties (Fincham, 1966; Hayes, 1964) of such complementation differ from those found when mutations in different cistrons are involved. Firstly, only special pairs of mutants will complement. The majority of mutants are non-complementary with others at the same locus. This behaviour occurs with point mutations and so cannot be due to deletions covering the locus. The mutational sites of the non-complementary mutants may be scattered over the whole locus, so

that recombination and complementation maps do not coincide. Secondly, the level of enzyme activity formed by interallelic complementation is always low and rarely exceeds 25% of the activity found in wild type strains or as a result of complementation between different cistrons. Thirdly, the enzyme formed is often, perhaps always, qualitatively different from wild type enzyme, for instance in temperature lability, and is also distinguishable from the relatively inactive enzyme synthesised by the complementing mutants alone. Fourthly, the derangement of synthesis of protein in each mutant is not severe; for instance, they may be "leaky" continuing to produce enzyme of very low activity, or they may make an inactive but serologically cross-reacting protein (CRM^+).

Interallelic complementation has been explained by the formation of functional protein by aggregation of two or more polypeptide subunits of the same type. Pairs with non-overlapping defects may be able to compensate for each other and form a "hybrid" structure which is stable and has some enzymic activity, but is different from the wild type. Other pairs have overlapping defects and cannot compensate. The absence of complementation among all the mutants of one gene suggests that the enzyme consists of only one polypeptide chain. This explanation allows for the fact that the mutational sites of non-complementary mutants may be distributed over the locus.

Complementation tests are tests of phenotypic function and can provide both genetic and functional information where the activity of the product of a mutated gene is measured directly and

quantitatively, but interpretation is not so simple where the activity being measured is not quantitatively graded, or the molecular mechanism is not known. For instance the activity being measured may be an indirect effect of the mutations involved, such as the end product of a multistep pathway for which the changes involved are in earlier steps. In such cases interpretation of a complementation test requires further information from tests with other pairs of mutants, recombination mapping, or qualitative data on, say, the stability of the activity.

In this thesis, constitutive is used to mean "non-inducible" following the terminology of Collins, Mandelstam, Pollock, Richmond & Sneath (1965), and the types of constitutive mutation are classified in the manner proposed by these authors. This is based on the basal and induced levels of enzyme activity made under standard conditions, and the induction ratio (I.R.) which is defined as the ratio of the differential rate of enzyme formation under defined standard conditions for maximal induction to the differential rate of enzyme formation without inducer. Loss of inducibility may be complete (I.R. = 1) as in the fully constitutive mutants, or partial (I.R. < wild type) as in semi-constitutive and semi-inducible mutants.

The relevant classes of fully constitutive phenotype are a) magno-constitutive: the enzyme level is that of the fully induced wild type strain (or usually slightly above this level); b) meso-constitutive: the enzyme level is greater than the wild type basal level but less than the fully induced amount;

c) baso-constitutive: the enzyme level is that of the uninduced wild type strain; d) micro-constitutive: the enzyme level is below that of the uninduced wild type strain. The partially constitutive phenotypes to be distinguished are a) semi-constitutive: the basal level of enzyme is greater than that of the wild type strain, and the induced level equal to that of the wild type strain; b) semi-inducible: the basal level is similar to the wild type strain, but the induced level is less than the normal induced amount.

All of these constitutive phenotypes have been found for the normally inducible penicillinase after mutation of S. aureus (Novick, 1963; Richmond, 1965c). The wild type strains make 10 units or less of penicillinase per milligram dry weight of bacteria uninduced and 300 units/mg. induced and have an I.R. of 30-100. For practical purposes the convention of Novick (1963) has been followed in this thesis and an I.R. of less than 5 is taken as defining a fully constitutive phenotype, sub-classification being made on the uninduced enzyme level, the magno-constitutive typically making 350 units/mg. Some of these phenotypes may arise by double mutation of both a regulator gene and of the structural gene for the enzyme, but they can also be found in cases where the structural gene appears intact and are hence solely control region mutations (Richmond, 1965c). The protein made by a structural mutant is called a mutein (Collins et al., 1965). Mutants making more basal enzyme than the uninduced wild type level are referred to as derepressed.

In the first part of the work described below, a series of haploid mutants of the wild type Y-plasmid were selected for derepressed enzyme levels, and classified phenotypically. This plasmid was used as it determines A-type penicillinase and mutations affecting the structure of this enzyme can be detected antigenically. The magno-constitutive mutant was shown to be repressed in the presence of an i^+ allele in the trans position, confirming the dominance of i^+ over i^- shown by Richmond (1965b). The meso-constitutive mutation was examined by its complementation behaviour in diploids, and by recombination, for information about its genetic constitution. A constitutive mutation, insensitive to repression, similar to those interpreted as defining the operator locus of β-galactosidase in E. coli (Jacob & Monod, 1961), was sought by mutation of a strain of S. aureus diploid for the penicillinase region. In the third section of the experimental work, an existing micro-constitutive strain was investigated to determine its genotype using mutational, recombinational and complementation data.

II. MATERIALS AND METHODS

Few of the methods described in this section were new or were developed by the writer, but were the standard methods used by Drs. M.R. Pollock, R.P. Novick, M.H. Richmond and others in investigations of penicillinase. Where possible, formal references are given, but in many cases the details have not been printed.

1. ABBREVIATIONS

7-ACA	7-aminocephalosporanic acid
6-APA	6-aminopenicillanic acid
CHY	casein hydrolysate yeast extract liquid medium
CRM	immunologically cross-reacting material
CY	casein hydrolysate solid medium
EMS	ethylmethane sulphonate
I.R.	induction ratio
NG	N-methyl-n'-nitro-N-nitrosoguanidine
Penicillin	benzylpenicillin; or penicillin G
U	unit of penicillinase
U/mg.	unit(s) of penicillinase per mg. dry wt. of bacteria
u.v.	ultraviolet
$V_{max.}$	the maximum velocity obtained when the substrate concentration is high enough to saturate the enzyme.

Bacterial strains are normally referred to only by their laboratory numbers and genotypes.

2. MATERIALS

Benzylpenicillin (Crystapen G) and cephalosporin C (as the potassium salt) were gifts from Glaxo Laboratories Ltd., Greenford, Middlesex.

Methicillin (Celbenin) was a gift from Beecham Research Laboratories Ltd., Brockham Park, Betchworth, Surrey.

Erythromycin (base) was a gift from Abbott Laboratories Ltd., Queenborough, Kent.

Sodium-β-glycerophosphate was obtained from Cambrian Chemicals Ltd., Croydon.

Ethylmethane sulphonate was obtained from Eastman Kodak Co., Kirkby Trading Estate, Liverpool.

N-methyl-N'-nitro-N-nitrosoguanidine was obtained from Koch-Light Laboratories, Colnbrook, Bucks.

3. STRAINS AND NOMENCLATURE

The strains of S. aureus used for initiating these experiments came from the culture collection of Dr. M.H. Richmond, and are listed in Table 2. The strain 8325-18 (referred to hereafter by the laboratory name P_o) was made by transduction of the α-plasmid from strain 524SC (Rogers, 1953) into the plasmid-negative strain NCTC 8325 (Novick, 1963; Novick & Richmond, 1965). Strain 147 (Segalove, 1947) normally carried the β-plasmid and strain MS 258 (Mitsuhashi, Morimura, Kono & Oshima, 1963) carries the γ-plasmid. The laboratory numbers of these strains and of those derivatives of them, which were obtained from Dr. Richmond, are shown in the Table. The genotypes, where known, are also shown in Table 2. The characteristics and nomenclature of mutants and recombinants of these strains obtained in the course of the experimental work are shown in the relevant part of the text. The genetic nomenclature is based on that of Novick & Richmond (1965). The host organism is distinguished from the plasmid by retaining the original strain number of the host and inserting after a colon a representation of the genotype of the plasmid involved. The greek letter refers to the plasmid as a whole and is followed by the letters and subscript figures indicating the markers which the plasmid carries and which are pertinent to the experiment under consideration. Strains that carry no penicillin plasmid and that have arisen as plasmid-loss variants of penicillinase-positive cultures have the letter N suffixed to their strain number, e.g., 147 N.

Table 2. The strains of *S. aureus* used for the initiation of the investigation.

Laboratory Number	Host strain	Plasmid and genotype of the penicillinase region (where known)	Derivation*	Source ≠	Penicillinase: Type	Penicillinase: Activity (U/mg. dry wt.) Uninduced	Penicillinase: Activity (U/mg. dry wt.) Induced	Penicillinase: Extracellular fraction (%)	Other genetic markers of plasmid ≠: Hg	Cd	Em	Compatability group
P_o		$\alpha\ i^+\ p_A^+$	Txn	(2)	A	10	300	45	R	R	S	I
P_o Hg^S Em^S		$\alpha\ i^+\ p_A^+$	EMS	(5)	A	10	300	45	S	R	S	I
P_o Hg^S Em^R		$\alpha\ i^+\ p_A^+$ --- γ	Rec	(5)	A	10	300	45	S	R	R	I
P_o Cd^S Em^R		$\alpha\ i^+\ p_A^+$ --- γ	EMS	(5)	A	10	300	45	R	S	R	I
PC1		$\alpha\ i^-\ p_A^+$	EMS	(2)	A	400	400	45	R	R	S	I
PC1 Cd^R Em^R		$\alpha\ i^-\ p_A^+$ --- γ	Rec	(5)	A	400	400	45	R	R	R	I
PC1 Cd^S Em^R	8325	$\alpha\ i^-\ p_A^+$ --- γ	Rec	(5)	A	400	400	45	R	S	R	I
P2i		$\alpha\ i^+\ p_{A2}^-$	EMS	(2)	A2	0·3	15	45	R	R	S	I
P2i Hg^S Em^R		$\alpha\ i^+\ p_{A2}^-$ --- γ	Rec	(5)	A2	0·3	15	45	S	R	R	I
P2i Cd^S Em^R		$\alpha\ i^+\ p_{A2}^-$ --- γ	Rec	(5)	A2	0·3	15	45	R	S	R	I
P2C Cd^R Em^S		$\alpha\ i^-\ p_{A2}^-$	Rec	(4)	A2	14	16	45	R	R	S	I
P16		N	EMS	(2)	-	-	-	-	S	S	S	-
P45 Cd^R Em^S		α ( )	EMS	(2)	(A)	0·6	1·2	45	R	R	S	I
P47		α ( ) P_A^+	EMS	(2)	A	7	9	45	R	R	S	I

Table 2 [Contd.]

258	258	$\gamma\ i^+\ p_A^+$	(1)	(1)	A	15	250	25	R	R	R	I
147i		$\beta\ i^+\ p_C^+$	(1)	(6)	C	10	250	5-10	R	R	S	II
147C223	147	$\beta\ i^-\ p_C^+$	EMS	(4)	C	250	250	5-10	R	R	S	II
147 N		N	(2)	(3)	-	-	-	-	S	S	S	-

* Derivations of strains are:

(1) naturally occurring
(2) spontaneous loss of plasmid
Txn - transduction
Rec - recombination
EMS - ethylmethane sulphonate-induced mutation

≠ (1) Mitsuhashi et al. (1963)
(2) Novick (1963)
(3) Novick & Richmond (1965)
(4) Richmond (1966a)
(5) Dr. M.H. Richmond
(6) Segalove (1947)

≠ Hg = mercuric ion) R and S indicate resistance or
Cd = cadmium ion) sensitivity of the strain (see text)
Em = erythromycin)

In cases where recombination has occurred between different plasmids, the designation indicates which of the markers in a recombinant comes from each of the parent plasmids, as far as can be ascertained. Thus 8325: $\underline{\alpha}\ \underline{i}^{+}\ \underline{p}_{A}^{+}$ --- $\underline{\gamma}\ \underline{Em}^{R}$ would designate genetically strain 8325 that carries a recombinant element the penicillinase region being that of $\underline{\alpha}$ and the erythromycin resistance marker contributed by $\underline{\gamma}$. Since all the erythromycin-resistant strains used have been obtained by recombination of an $\underline{\alpha}$-plasmid either with the $\underline{\gamma}$-plasmid directly or with an $\underline{\alpha}$ --- $\underline{\gamma}$ recombinant, all these strains contain an $\underline{\alpha}$ --- $\underline{\gamma}$ recombinant plasmid. In plasmid hetero-diploids, the respective plasmid genotype designations are separated by an oblique stroke, e.g. 147: $\underline{\alpha}\ \underline{i}^{+}\ \underline{p}_{A}^{+}/\underline{\beta}\ \underline{i}^{+}\ \underline{p}_{C}^{+}$.

The symbol $\underline{p}$ in the plasmid genotypes refers to the structural gene for penicillinase and $\underline{i}$ to a penicillinase inducibility locus. The two immunological types of penicillinase, A and C, determined by variants of the structural gene, $\underline{p}$, are indicated by p_A and p_C. The other plasmid markers are designated $\underline{Hg}$, $\underline{Cd}$, and $\underline{Em}$ for the loci for mercury, cadmium and erythromycin resistance. The superscript letters R and S indicate the resistant and sensitive forms respectively. In wild type plasmids there is probably no allele for $\underline{Em}^{S}$ but a deletion for this region (Novick, 1967a).

The phages used in transduction are temperate phages carried by the donor strain (8325 in most cases), and were induced by ultra violet (u.v.) irradiation (see Section II.10 below). The indicator

strain NTCT 1030, kindly provided by Dr. E.H. Asheshov, was used for the titration of phage suspensions.

4. MEDIA

Media of the following composition have been used throughout.

(a) CHY liquid medium (Novick, 1963).

Casamino acids (Difco)	1·0% (w/v)
yeast extract (Difco)	1·0% (w/v)
sodium β-glycerophosphate	0·12 M (3·8% w/v)
$MgSO_4.7H_2O$	0·001 M (0·025% w/v)
trace metal solution	0·02 ml./l.

The mixture was autoclaved in 500 ml. lots, and then sterile glucose (1·6% w/v; 20 ml. 40% w/v per 500 ml.) was added. This medium is buffered at pH 7·4 by the sodium β-glycerophosphate

(b) CY agar solid medium (modified from Novick, 1963).

Casamino acids (Difco)	0·5% (w/v)
yeast extract (Difco)	0·1% (w/v)
$MgSO_4.7H_2O$	0·001 M (0·025% w/v)
trace metal solution	0·02 ml./l.
agar (Difco, Bacto-)	2·0% (w/v)
or agar (New Zealand)	1·5% (w/v)

The mixture was autoclaved in 500 ml. lots. Before use the following separately-sterilised ingredients were added:

sodium β-glycerophosphate	0·06 M (20 ml. 1·5 M)
glucose	1·6% (20 ml. 40% w/v)
starch (soluble)	0·012% (20 ml. 3% w/v)

The quantities shown were for each 500 ml. lot.

Trace metal solution (Novick, 1963; Richmond, 1963).

$CuSO_4.5H_2O$	0·5% (w/v)
$ZnSO_4.7H_2O$	0·5% (w/v)
$FeSO_4.7H_2O$	0·5% (w/v)
$MnCl_2.4H_2O$	0·2% (w/v)
Conc. HCl (12 N)	10% (v/v)

The following media were used for the maintenance of stock cultures.

(c) Semi-solid agar deeps

Nutrient broth No.2 (Oxoid) 100 ml.

"Ionagar" No.2 (Oxoid) 0·6 gm.

Autoclaved in 5 ml. lots in bijoux bottles.

(d) Nutrient agar slopes

Nutrient agar CM3 (Oxoid)

Autoclaved in 3 ml. lots in bijoux bottles and slanted while setting.

In tests for mercury sensitivity peptone agar was used.

(e) Peptone agar (Moore, 1960)

Peptone (Evans)	2%
NaCl	0·5%
"Ionagar" No.2	1%

Adjusted to pH 7·4, and autoclaved.

(f) Additives for screening, selection and induction (including mercury discs).

In order to select or screen clones for resistance properties the following additions were made to the medium.

(i) Erythromycin

A fresh solution of erythromycin at 1 mg./ml. was prepared aseptically, with grinding if necessary for dissolution, in sterile CHY, and added (1/100 (v/v)) to molten CY agar immediately before use to a final concentration of 10 µg./ml.

(ii) Cadmium ions (R.P. Novick, personal communication)

A sterile stock solution of cadmium acetate or chloride at 10^{-2} M in distilled water was prepared and autoclaved. This was diluted 1/100 in molten CY agar to give a final concentration of 10^{-4} M.

(iii) Mercury ions (Moore, 1960; Green, 1962)

A stock solution of 10 mg. $HgCl_2$/ml. was prepared aseptically in sterile distilled water, heated as necessary to help dissolution.

The solution was added to Peptone agar to give a final concentration of 45 - 75 µg. $HgCl_2$/ml. The optimum concentration for differentiation of sensitive and resistant strains varied for each batch of medium and was determined from a test series of dilutions inoculated with standard sensitive and resistant strains on each occasion that a new batch of medium was made.

(iv) Mercury discs (Moore, 1960)

Phenyl mercuric nitrate 0·1% (w/v) was dissolved in hot absolute alcohol and diluted in hot alcohol to 1/150,000 (w/v). Amounts of 0·02 ml. were distributed on 0·6 cm. filter paper discs. (Whatman No.1). The discs were dried at room temperature overnight, and stored in a dry place until required.

For the induction of clones grown on solid media the following addition was made to the medium.

(v) Cephalosporin C

A solution of Cephalosporin C (1 mg./ml.) was prepared aseptically in sterile distilled water. A fresh solution was diluted into molten CY agar to 10 µg./ml. final concentration immediately before use.

5. GROWTH CONDITIONS AND MAINTENANCE OF CULTURES

(a) Incubation

All cultures were incubated at 37°. Liquid cultures were grown at 37° in conical flasks aerated by shaking on a graded shaker. (Kantorowicz, 1951).

(b) Bacterial culture density

The densities of bacterial suspensions were determined by measurement of the optical density at 675 mµ (Unicam Model SP 600 spectrophotometer), and converted to mg. dry wt. bacteria/ml. or to number of bacteria/ml. by reference to a standard curve.

(c) Tests and selection for resistance markers

Cadmium and erythromycin resistance were both tested for by plating strains on CY agar containing the appropriate additives; visible growth signified resistance. Growth was relatively unaffected by the inoculum density. These two media are also suitable for selecting resistant clones from a dense background population, e.g., from a transduction experiment, or for

distinguishing sensitive and resistant clones by replica plating on to them.

Mercury resistance was tested either by streaking a loop-ful of a fresh culture at about 10^8 organisms/ml. on peptone agar containing mercury and comparing the growth of test strains with that of sensitive and resistant controls, or by comparing the size of the zone of inhibition around a mercury disc with the zones obtained with the control strains on the same peptone agar plate. This marker is unsuitable for use in isolating resistant strains since the difference in mercury concentration inhibiting resistant and sensitive strains is low, and survival is influenced by the inoculum size, but differentiation between sensitive and resistant clones was usually possible after replica plating onto peptone agar at two levels of $HgCl_2$.

(d) Maintenance of cultures

Cultures grown on the surface of CY agar were kept for up to three months at 4°. Stocks kept for longer periods were grown both in semi-solid agar deeps and on nutrient agar slopes in bijoux bottles and stored at room temperature. The lids of the bijoux bottles were closed tightly to prevent desiccation.

6. INDUCTION OF PENICILLINASE

Cultures in liquid medium were routinely induced with methicillin (see Section II.7.(c).(ii)), but on some occasions cephalosporin C was used as inducer (see text). Cultures grown

on solid medium were induced by cephalosporin C included in CY agar medium.

7. ESTIMATION OF PENICILLINASE ACTIVITY

(a) Unit of penicillinase activity

1 unit U of penicillinase is defined as equal to the enzymic activity by which 1·0 μmole of penicillin G is destroyed per hour at 30° and pH 5·9 (Pollock & Torriani, 1953; modified by Novick, 1962a).

(b) The Perret assay (Perret, 1954; Novick, 1962a)

(i) Reagents

0·1 M-phosphate buffer pH 5·9

K_2HPO_4	17·4 gm.
KH_2PO_4	122·5 gm.
distilled water to	10 litres

2 M-acetate buffer pH 4·0 - 4·2

glacial acetic acid	170 ml.
sodium acetate anhydrous	86·6 gm.
or sodium acetate hydrate	143·5 gm.
distilled water to	2 litres.

Stock iodine

iodine	8·12 gm. (0·32 N)
potassium iodide	40·0 gm. (1·2 M)
distilled water to	200 ml.

This solution was stored in a brown bottle.

Iodine in acetate buffer

The stock iodine solution was diluted 1/20 in acetate buffer for use in the assay at 0·016 N-iodine and 0·06 M-KI.

Stock thiosulphate

sodium thiosulphate	41·200 gm. (0·166 N)
distilled water to	1 litre

0·0166 N-thiosulphate

The stock solution was diluted accurately 1/10 for use in the titration.

(ii) Assay procedure

A benzylpenicillin solution was prepared by dissolving 10^6 units of penicillin in 250 ml. of phosphate buffer pH 5·9. Portions of 5 ml. (2×10^3 units) were placed in 50 ml. conical flasks and warmed in a water bath at 30°. Enzyme was added to a flask and the reaction allowed to continue for an exact time. The reaction was stopped by adding 10 ml. of iodine in acetate buffer. A control flask of 5 ml. of penicillin at 30° was incubated during the same time and iodine added a minute later than to the test flask, followed by an equal quantity of enzyme material. Both flasks were incubated for a further 10 minutes at 30°, or longer if more convenient. The test and control were titrated at minute intervals against 0·0166 N-thiosulphate using starch as an indicator. With practice it was possible to complete about 12 estimations in 1 hr.

(iii) Calculation of penicillinase concentration

As 1 mole of penicilloic acid reduces approximately 8·3 equivalents of iodine, the equation relating penicillinase concentration to the titration reading is given by

$$[E] = \frac{2\ (V_o - V)}{a.t/60}$$

Where [E] is the penicillinase concentration in U/ml.

V, V_o are the test and control titration readings

a is the volume assayed (ml.)

t is the time of assay (min.).

(c) Preparation of cultures for assay (Richmond, Parker, Jevons & John, 1964)

(i) Uninduced cultures

An overnight broth (CHY) culture was diluted 1/100 in CHY medium, and incubated for 4½ hr. with shaking. Portions estimated to give a titration difference of 1 - 6 ml. in the chosen time were assayed by the Perret method. The enzyme concentration (U/ml.) was divided by the bacterial concentration (mg. dry wt./ml.) to get the penicillinase activity characteristic of the strain (U/mg.). Cultures awaiting assay were kept in an ice-bath at 4°.

(ii) Induced cultures

Cultures were set up as for the uninduced cultures, except that after 1 hr. incubation methicillin was added at 0·5 µg./ml. Incubation was continued for a further 3½ hr. and the cultures were assayed similarly to the uninduced cultures.

(iii) Exoenzyme (Novick & Richmond, 1965)

Exopenicillinase measurements were made on the supernatants of uninduced and induced cultures, grown under the standard conditions, and centrifuged for 10 min. at 8,000 g at 4°. The supernatants were kept at 4° until assayed by the Perret assay. Exoenzyme was usually expressed as the percentage of penicillinase in each unit of supernatant divided by the units of penicillinase in the same volume of the whole culture.

(d) Detection of penicillinase in colonies grown on solid medium (modified from Perret, 1954)

Cultures were grown on CY agar containing the indicator starch (0·012% w/v). A penicillin-iodine reagent was prepared to contain benzylpenicillin (5×10^4 units/ml.) in phosphate buffer pH 5·9 and a suitable strength of iodine. The iodine concentration was varied from 1/4 to 1/50 of the stock iodine solution (for Perret assay) depending on the expected activity of the culture. The culture plate was flooded with the penicillin-iodine reagent for a suitable time, usually about 1 min. and the excess poured off. The agar turned deep blue and the colonies yellow-brown. Penicillinase-positive colonies decolourised and a colourless zone, increasing with time, appeared in the medium around them. The rate of decolourisation was proportional to the penicillinase activity so that comparative estimates of the enzyme level could be obtained. A positive result can be found for colonies with as little penicillinase as 0·05 U/mg. dry wt. of organisms (Novick & Richmond, 1965).

This method, outlined in the previous paragraph, was used for screening single colonies and a rough comparison of the level of enzyme production was possible allowing detection of mutants, recombinants or segregants. Alternatively, rapid screening of a number of strains and grading them in relation to known strains was possible by plating uniform streaks or patches on the same plate as the known strain. An indication of the induced level of penicillinase could be obtained by including cephalosporin C in the medium (see Section II.4.(f).(v)).

(e) Accuracy of penicillinase determinations

Although the actual Perret assay gave extremely reproducible results (± 0·5 U/ml. for cultures 0 - 10 U otherwise less than ± 5%) when repeated analyses are performed on identical samples, the other stages in the determination of the enzyme production of a particular strain were not accurately reproducible. Richmond et al. (1964) reported that reproducibility was about ± 25%, and this was in general agreement with my own observations. The reproducibility for basal enzyme levels has normally been better (± 20%), but measurements of induced levels, and hence induction ratios may vary almost to ± 50%, as shown in the case of mutant K19 (see Table 5, Section III.5.(a)). Causes of this inaccuracy are probably due in part to errors from the O.D. measurement arising from differential clumping, cell lysis, etc.; the phase of growth reached by the culture; and variations between batches of media.

8. SEROLOGICAL EXAMINATION OF PENICILLINASE (Richmond, 1963, 1965a & b)

(a) Antiserum

The antiserum prepared by Richmond (1963) to purified A-type penicillinase was used. This serum stimulates the enzymic activity of type A penicillinase approximately 4 - 5 fold, but the activity of type C enzyme is virtually unaffected by the antiserum. With mixtures of A- and C-type enzymes, the presence of the latter does not alter the degree of stimulation of the A-type component (Richmond, 1965b). It is therefore possible to estimate the quantity of A-type enzyme in such a mixture.

(b) Method for antigenic determinations

Serological reactions were studied with samples of exo-penicillinase diluted in CHY to give a titration difference in the Perret assay of about 1 ml. when 0·5 - 1·0 ml. samples were used in the test. A series of flasks were prepared, each with a sample of the enzyme for examination (constant antigen). Appropriate quantities of antiserum diluted 1/10 in 0·15 M-NaCl (usually 1/1000 to 1/100 ml. of the original serum) were added, and the mixtures incubated 15 - 30 min. at room temperature. The enzymic activities were then assayed by the Perret assay. The reaction was begun by adding 5 ml. of a warmed penicillin solution to the flask. Control flasks without enzyme were set up at minute intervals from the test flasks and the enzyme was added after the iodine in acetate buffer. Measurements on a similar amount of A-type enzyme alone were included for comparison, as the absolute

amount of stimulation is liable to vary slightly from day to day (Richmond, 1965b).

(c) Interpretation of antiserum experiments

The antigenic reaction of an enzyme solution can be used to investigate two factors: (i) it can show qualitative changes in the enzyme protein; and (ii) quantitative estimations can be made of the amount of A-type enzyme present in a solution containing both A and C penicillinase.

The point of maximum stimulation of activity is called the protein-antibody equivalence point (E in Fig. 3), and for a given amount of enzyme is determined by the nature of the enzyme protein. The rate of increase in enzymic activity with increasing antibody concentration (given by the slope of the graph of total activity against the amount of serum added between O and E, Fig. 3), are therefore standard for normal A-type enzyme protein and can be used for qualitative evaluation of enzyme from other strains. In practice, the exoenzyme made by a mutant strain was examined serologically in the same experiment as a standard A penicillinase preparation, and the slopes of the lines showing the rate of increase in activity for each enzyme were compared. The mutant strain was considered to be synthesising unmutated A-type enzyme when this slope was the same as that obtained with the control preparation. Mutein enzyme (CRM^+) might be altered in the affinity of the protein for the antibody, the extent of stimulation of activity, or the specific activity of the enzyme protein (Pollock, 1964). A result of these mutations would be a change in the position of the equivalence point and the

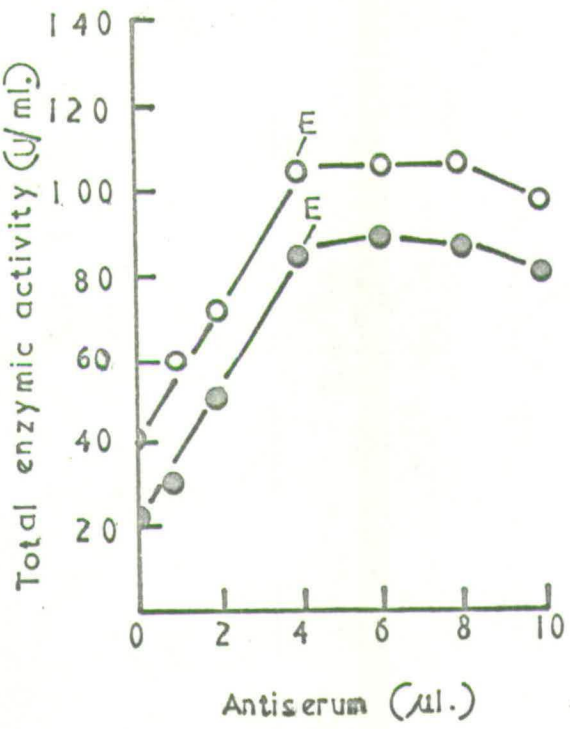


Fig. 3. (From Richmond, 1965b)

Reaction of antiserum, prepared against purified A-type penicillinase, with mixtures of A- and C-type (O) penicillinase. The reaction against A-type enzyme alone (●) is also shown. E indicates the equivalence point. Experimental details are given in the text.

rate of stimulation of activity. Two muteins (A_2 and A_{54}) that have been purified retain the protein and enzymic reactions with antiserum, but have a different equivalence point determined by their specific activity (Richmond, 1965b; and personal communication). In these cases comparison of the line showing the rate of increase in activity with the slope obtained with wild type enzyme indicates the relative activity of the mutein.

At the equivalence point (E), both the serum concentration and the total activity are proportional to the initial activity for a given enzyme preparation. As Richmond (1965b) found that the total stimulation for A-type enzyme is unaffected by the presence of C-type enzyme, the total activity at the equivalence point can be used to estimate quantitatively the amount of A-type penicillinase present in a sample (such as exoenzyme from a diploid strain) also containing C-type penicillinase. A known amount of standard A-type enzyme was treated with antiserum and the stimulation factor, given by the total activity at E divided by the initial activity, was found for this enzyme type for the same conditions and day of assay as the unknown sample. The amount of A-type enzyme in the sample was calculated from the equation

$$\frac{[E_A]}{[E_T]} = \frac{\mu' - \mu_o}{(F-1)\mu_o}$$

where

$[E_A]$ is the concentration of A-type enzyme in the sample

$[E_T]$ is the total enzyme concentration in the sample

μ' is activity at E) in a given volume of
μ_o is initial activity) the sample
F is the stimulation factor for standard A-type enzyme.

9. MUTATION

(a) Ethylmethane sulphonate (EMS) (Loveless & Howarth, 1959; Novick, 1963)

An overnight broth culture was diluted 1/50 into fresh liquid CHY medium, incubated at 37° with shaking until the cell density was 0·4 mg. dry wt./ml. (approximately 2 hr.). A sample (4·0 ml.) of the whole culture was added to 0·16 ml. EMS, which was dissolved by gentle pipetting or by vortex mixing. The bacterial suspension was incubated at 37° for 20 minutes without shaking, centrifuged, and the cells washed once in 4 ml. of 0·15 M-NaCl, and resuspended in 50 ml. CHY. This culture was incubated until the optical density reading had doubled (about 4 hr.),or overnight (18 - 24 hr.). The bacteria were plated on CY agar (with additions for selection when indicated in the text), at dilutions calculated to give 500 to 2,000 colonies per plate.

(b) N-Methyl-N'-nitro-N-nitrosoguanidine (NG) (Dubnau & Pollock, 1965)

An overnight broth culture, average density 3 - 5 mg. dry wt./ml., of stationary phase cells, was centrifuged and resuspended in the same volume of 0·2 M-sodium acetate buffer pH 5·0. Ng (4·0 mg.) was dissolved in 1·0 ml. of sodium acetate buffer pH 5·0, membrane (Oxoid) filtered, and 0·12 ml. added to 0·5 ml. of the bacterial

suspension. This suspension was incubated at 37°, without shaking, for 210 min., and diluted 1/30 into 15 ml. of fresh CHY medium. The bacteria were grown overnight at 37°, diluted and plated for the isolation of mutants.

(c) X-ray irradiation (Glover, 1956)

An overnight broth culture was diluted 1/50 into fresh CHY medium and incubated about 2 hours to obtain log phase growth. This culture was centrifuged and resuspended in 1/10 of the volume of fresh CHY so as to give about 5×10^9 bacteria/ml. or about 3 mg. dry wt./ml.. A portion (6 ml.) of this suspension was placed in a 5 cm. diameter Petri dish with a glass cover and centred on a wooden block at 20 cm. from the source of X-rays. The radiation intensity was 308 roentgens (r.)/min. at 20 cm.. The doses used were 23,100 r. (75 min.), 36,960 r. (120 min.), 55,440 r. (180 min.), and 73,920 r. (240 min.).

After irradiation 2·5 ml. samples were removed and diluted into 50 ml. of CHY. Samples were further diluted and plated immediately for a control of the viable count. The remainder was incubated for 3 - 4 hr. until definite growth was evident from the optical density value, and then diluted and plated for mutant selection.

(d) Isolation of mutants

After all mutagenic treatments, the colonies picked from the initial plating underwent three sequential single clone isolations before the isolate was examined further.

Mutants of the penicillinase region were isolated by screening

the whole population of colonies with the penicillin-iodine reagent (Section II.7.(d) above). Although selection on the basis of differences in the degree of penicillin resistance, due to the amount of penicillinase synthesised, is possible using certain combinations of strains (Novick, 1963), it is not a feasible method for the isolation of mutants because of the small differences in the levels of penicillinase made by some mutants, and because of the density of satellite growth of non-mutant strains around colonies of greater levels of synthesis due to destruction of the selecting agent (penicillin).

10. TRANSDUCTION

The method was essentially that of Novick (1963).

(a) Preparation of transducing phage

A broth culture of the strain to be used as the donor is diluted about 1/50 in fresh CHY medium and grown to mid-log phase (about 2 hr.). The cells were spun down and resuspended in 0·15 M-NaCl to a density of 0·5 mg. dry wt./ml. Portions (2 ml.) were spread evenly in a clean, grease free, glass Petri dish (4 inches in diameter) and exposed to the u.v. source (Hanovia Lamp, model 11, high pressure mercury arc) at about 30 cm. from the source for 1 - 2 seconds. A sample (1·5 ml.) of the suspension from the Petri dish was pipetted into 1·5 ml. of CHY medium in a sterile test tube and incubated for 3 to 4 hours or until lysis occurred. The lysate was centrifuged at 6,000 g. for 10 min. at

room temperature to remove cell debris. The supernatant was freed of bacterial contamination by filtration through a membrane filter (Oxoid) at 4 - 5 lb./sq. in. air pressure. Phage lysates have been kept for up to 12 months at 4° without significant loss of titre. Phage titres of 10^8 - 10^9/ml. were obtained when titrated using the universal indicator strain, NTCT 1030.

(b) Transduction procedure

Transduction was carried out in sterile centrifuge tubes. The recipient strain was grown in CHY medium for 2 - 3 hr. to attain a log phase culture at a density of 5 x 10^8 to 10^9 bacteria/ml. A sterile solution (0·1 ml.) of 0·012 M-$CaCl_2$ and 0·2 ml. of a sterile solution of 40% (w/v) glucose was added to 2 ml. of the phage suspension in a sterile centrifuge tube. Finally, not more than 0·2 ml. of the recipient culture was added to the phage, so that the final bacterial concentration was 5 x 10^7 - 10^8/ml. As a control, a tube containing 2 ml. of CHY in lieu of phage was set up at the same time.

Transduction and control tubes were incubated at 37° for 50 min. to allow phage absorption. The bacteria were removed by centrifugation at 6,000 g. for 10 min., and the supernatant containing unadsorbed phage was discarded. The cells were re-suspended in 2 ml. of saline for immediate plating of 0·1 ml. portions on suitable selective media; or the cells were resuspended in a suitable volume of CHY and incubated further (4 - 24 hr.) to allow plasmid segregation before plating on the selective medium. The control tube was treated in the same way and portions from this

tube and from the original phage suspension were also plated on the same selective medium as a check that colonies obtained were, in fact, transductants.

(c) Selection of transductants

Transductants were selected for erythromycin resistance or for cadmium resistance by plating on CY agar containing the appropriate additive. In some cases transductants could be selected for both these markers at the same time. Mercury resistance and penicillin resistance were not used for selection as selection is difficult with these markers (as discussed in Sections II.5.(c) and II.9.(d)). All transductant colonies were submitted to three sequential single colony isolations after the initial isolation before being studied.

11. RECOMBINATION (Novick, 1963; Novick & Richmond, 1965)

(a) Procedure

Recombination experiments were performed by transduction of an $\underline{\alpha}$ or $\underline{\alpha}$ --- $\underline{\gamma}$ recombinant plasmid into a strain already carrying an $\underline{\alpha}$ or an $\underline{\alpha}$ --- $\underline{\gamma}$ plasmid. The transduction procedure is given in Section II.10. After the absorption of the phage, the recipient bacteria were incubated in fresh CHY medium for times varying from 4 to 24 hr. to allow segregation to occur. Clones receiving donor genetic material were selected by a resistance property carried on the donor plasmid. Erythromycin resistance was usually used for this purpose. Recombinants in the penicillinase region were scored

by staining with the penicillin-iodine reagent in the usual way. The recombination rate was expressed as a proportion of the total number of transductants (Richmond, 1966a).

(b) Discussion of the recombination rate

The manner of expressing the number of recombinants as a proportion of the total number of clones receiving a linked resistance marker, appears similar to the expression for the recombination rate from a three factor cross. However, the donor plasmid can determine its own replication and the selection method does not discriminate between strains carrying the whole plasmid and recombinants of it. Thus strains in which replacement of the whole plasmid has occurred might be represented in the total number of transductants. This situation is, strictly, one in which two complete chromosomes are involved in recombination (analogous to the situation with phage recombination), and one parental type is being selected with the recombinants. Comparison of recombination rates is thus of doubtful validity and was restricted to crosses in which the transductants were selected in the same way and if possible in the same recipient strain. Another complication that would affect the results is the possibility that the plasmid is a small circular linkage group (Stahl & Steinberg, 1964; Hopwood, 1965).

(c) Standard crosses between other plasmid markers

One of the following standard crosses was used to obtain plasmids carrying certain mutants of the resistance markers outside the penicillinase region.

(i) To obtain the markers $\underline{Hg}^S\ \underline{Em}^R$ the donor strain was P2i $\underline{Hg}^S\ \underline{Em}^R$. This strain had a small penicillinase activity and the plasmid genotype $\underline{\alpha}\ \underline{i}^+\ \underline{p_A}2^-\underline{Hg}^S$ --- $\underline{\gamma}\ \underline{Em}^R$. The recipient strains for this cross made derepressed levels of penicillinase and carried the plasmid $\underline{\alpha}\ (\underline{i}\ \underline{p_A})\ \underline{Hg}^S\ \underline{Em}^S$. Transductants were selected for erythromycin resistance and recombinants with the genotype $\underline{\alpha}\ (\underline{i}\ \underline{p_A})$ $\underline{Hg}^S$ --- $\underline{\gamma}\ \underline{Em}^R$ were isolated on the basis of their derepressed levels of penicillinase synthesis.

(ii) To obtain the markers $\underline{Cd}^S\ \underline{Em}^R$ the donor strain was PC1 $\underline{Cd}^S\ \underline{Em}^R$. This strain made derepressed levels of penicillinase and had the plasmid genotype $\underline{\alpha}\ \underline{i}^-\ \underline{p_A}^+\ \underline{Cd}^S$ --- $\underline{\gamma}\ \underline{Em}^R$. The recipient strains for this cross had a small penicillinase activity and carried the plasmid $\underline{\alpha}\ (\underline{i}\ \underline{p_A})\ \underline{Cd}^R$ --- $\underline{\gamma}\ \underline{Em}^S$. Transductants are selected for erythromycin resistance, and recombinants with the genotype $\underline{\alpha}\ (\underline{i}\ \underline{p_A})\ \underline{Cd}^S$ --- $\underline{\gamma}\ \underline{Em}^R$ were isolated on the basis of their low levels of penicillinase synthesised.

12. DIPLOID STRAINS (Novick & Richmond, 1965)

(a) Construction of plasmid heterodiploids

Diploids were constructed by transduction of an $\underline{\alpha}$-plasmid or an $\underline{\alpha}$ --- $\underline{\gamma}$-plasmid into a strain already containing a $\underline{\beta}$-plasmid. The host strain is usually 147 though 8325 is also suitable. The plasmids used were such that, of the markers outside the penicillinase region, each plasmid carries one resistant allele for which the sensitive allele was carried by the other plasmid. Thus

in the earlier experiments, an $\underline{\alpha}\ \underline{Hg}^S$ --- $\underline{\gamma}\ \underline{Em}^R$ recombinant was transduced into a strain carrying a $\underline{\beta}\ \underline{Hg}^R\ \underline{Em}^S$ plasmid. While these experiments were in progress, a plasmid linked marker for resistance to cadmium ions was found (Novick, personal communication; Novick & Roth, 1967). After this discovery, heterodiploids were established with the plasmids $\underline{\alpha}\ \underline{Cd}^S$ --- $\underline{\gamma}\ \underline{Em}^R$ and $\underline{\beta}\ \underline{Cd}^R\ \underline{Em}^S$. Transductions were carried out in the usual manner (Section II.10) and transductants were selected either for the donor $\underline{Em}^R$ marker, or when appropriate for both the donor $\underline{Em}^R$ and the recipient $\underline{Cd}^R$ alleles.

(b) Confirmation of diploidy

If an isolate had a phenotype that required the presence of both the resistant alleles carried singly by the two parent plasmids it was presumed to be diploid. Diploidy was confirmed by isolating haploid segregants of both parental types. The presumptive diploid clone was grown in CHY medium until sufficient growth had occurred to permit measurement of the cell concentration (6 - 24 hr.), and suitable dilutions were plated on CY agar to give between 200 and 500 single colonies per plate. These colonies were tested for each parental resistance marker by replica plating, and segregants were isolated as singly resistant clones, usually present at about 0·1 - 1% of diploid cultures. Both segregant types were shown to have the appropriate parental properties for the penicillinase region as regards the basal and induced levels of enzyme activity and the type of enzyme synthesised, as well as the appropriate properties for the other plasmid markers. Evidence

for the type of enzyme synthesised was deduced from the proportion of enzyme that was extracellular, and, when necessary, the antigenic reaction.

When diploids had been constructed from plasmids with markedly different penicillinase properties, segregants were sometimes isolated using this characteristic.

(c) Properties of heterodiploids

Since the resistant form of the genes for resistance to erythromycin and metal ions is dominant over the sensitive alleles (Novick & Richmond, 1965; Novick, personal communication), the diploid strain exhibits the combined resistances of both parental strains. The penicillinase structural genes of both plasmids are expressed and each type is released into the medium as exoenzyme in the proportions characteristic for that type alone (A-type about 50%, C-type about 5 - 10% of the total activity) (Richmond, 1965b). The amount of type A penicillinase synthesised was estimated from the exoenzyme, either as the proportion of the total activity released or by serological analysis. If both structural genes are equally expressed then about 80 - 90% of the exoenzyme would be A-type penicillinase.

13. PURIFICATION OF STAPHYLOCOCCAL PENICILLINASE

Attempts were made to purify the protein made by the i gene mutant by the methods normally used to prepare penicillinase from the parent organism (Richmond, 1965a; R.P. Ambler, personal

communication). The procedure consisted of stirring cellulose phosphate (Whatman P-70) into cultures at the stage of maximal exoenzyme formation until 80% (by assay) of this enzyme had been bound, washing the cellulose phosphate in distilled water until there were no more cells in the washings, and packing it as a column. The diameter of the column was chosen such that the bed height was 5 - 10 times the diameter. The column was washed with 0·1 M-ammonium acetate pH 6·5, and the enzyme activity eluted with 75% saturated ammonium sulphate pH 7·5. The active eluate was dialysed against distilled water and then freeze-dried, before being fractionated by gel-filtration through Sephadex G-100 (in a column 2·5 cm. diameter x 100 cm., 0·05 M-ammonium acetate pH 5·0). After elution the 5 ml. fractions were examined for protein (by u.v. spectrophotometry) and assayed for enzyme activity.

Peptide and amino acid fingerprinting of a purified protein were carried out by high voltage electrophoresis and descending chromatography using the methods described by Ambler (1963).

EXPERIMENTAL RESULTS

III. THE HAPLOID MUTANTS

1. THE ISOLATION OF HAPLOID MUTANTS

The wild type haploid strain, (8325 carrying the plasmid $\underline{\alpha}\ \underline{i}^{+}\ \underline{p}_{A}^{+}\ \underline{Cd}^{R}\ \underline{Hg}^{S}\ \underline{Em}^{S}$) was treated with EMS and plated on CY agar. Derepressed mutants were picked on the basis of their increased level of penicillinase synthesis as detected by staining the colonies with penicillin-iodine reagent. This series of mutants were given the initial identification letter K and numbered in the order of their isolation. The isolates were assayed under standard conditions and then assigned to the various constitutive categories described in the Introduction (Section I), on the basis of their phenotypic properties (see Table 3). The induced levels of penicillinase obtained in these first assays tend to be systematically higher than those found later. The reason for this discrepancy is not known, but may have been due to technical reasons associated with the measurement of bacterial density of the cultures assayed.

Colonies with a raised basal level of enzyme expression were found at a frequency of 0·1 - 0·5% of the total count, which indicates a mutation rate of about 1×10^{-3}. Of the mutants picked, 4 were magno-constitutive, 8 were semi-constitutive, 5 were meso-constitutive, and 4 were semi-inducible. Baso- and micro-constitutives would not have been detected with this series.

To facilitate selection of transductants and segregants involving plasmids from these mutants, the erythromycin-resistance marker was recombined into them (for method see Section II.11.(c).(i)).

Table 3. The properties of haploid mutants of *S. aureus*; EMS-induced mutations in strain P_o(8325: *a* i^+ p_A^+ Hg^S Em^S) affecting penicillinase formation.

Strain	Phenotype	Penicillinase activity (U/mg.) Uninduced	Induced	Induction ratio
K3 (K4)*	magno-constitutive	512	596	1·2
K7 (K8)*		568	648	1·1
K11		>500	>500	-
K41		684	617	0·9
K9	semi-constitutive	297	531	1·8
K42		211	544	2·6
K40		197	492	2·5
K17 (K18)*		108	663	6·1
K21		91	436	4·8
K15		63	600	9·5
K39		47	479	10
K26 (K27)*		27	447	16
K14	semi-inducible	9	130	14
K34 (K35)*		7	71	10
K1		4	179	45
K2		4	34	8·5
K5	meso-constitutive	22	51	2·5
K13		38	75	2·0
K37		55	27	0·5
K19 (K23)*		80	161	2·0
K32		39	105	2·7
P_o(8325: *a* $i^+p_A^+$)	wild type inducible	5	438	88

* The mutants shown in brackets are the second clone of similar phenotype isolated from the same plate, and are therefore presumed to be sib isolates.

As the object of these experiments was ultimately to study the control of penicillinase synthesis, mutants altered solely in the structure of the penicillinase molecule were irrelevant, and had to be excluded. Any mutant strains in which enzyme activity can reach normal maximum levels are unlikely to be synthesising a mutated enzyme and are therefore suitable for study. The magno-constitutive mutant, K3, falls in this category and is presumed to carry a $\underline{p}^+$ gene. Meso-constitutive mutants, in contrast, cannot be induced to produce maximal levels of enzyme activity and may therefore be either a structural or a regulatory gene mutant, or both; for this reason the enzyme synthesised by the meso-constitutive strain, K19, was tested serologically for any evidence for the synthesis of an altered penicillinase molecule. The graph showing the stimulation of activity of exoenzyme from K19 with anti-A serum has a slope similar to that for wild type A penicillinase (Fig. 4). As the muteins that have been examined have been altered in this respect (M.H. Richmond, personal communication), it was assumed that K19 had a normal structural gene, $\underline{p}_A^+$, and was therefore suitable for study as a mutant of a regulatory region.

2. MAGNO-CONSTITUTIVE MUTANTS

The first class of regulatory mutant examined are the magno-constitutives. Mutants of this type have been shown previously (Richmond, 1965b & c) to be *trans* recessive to an $\underline{i}^+$ allele in diploids and after induction of the diploid strain both structural

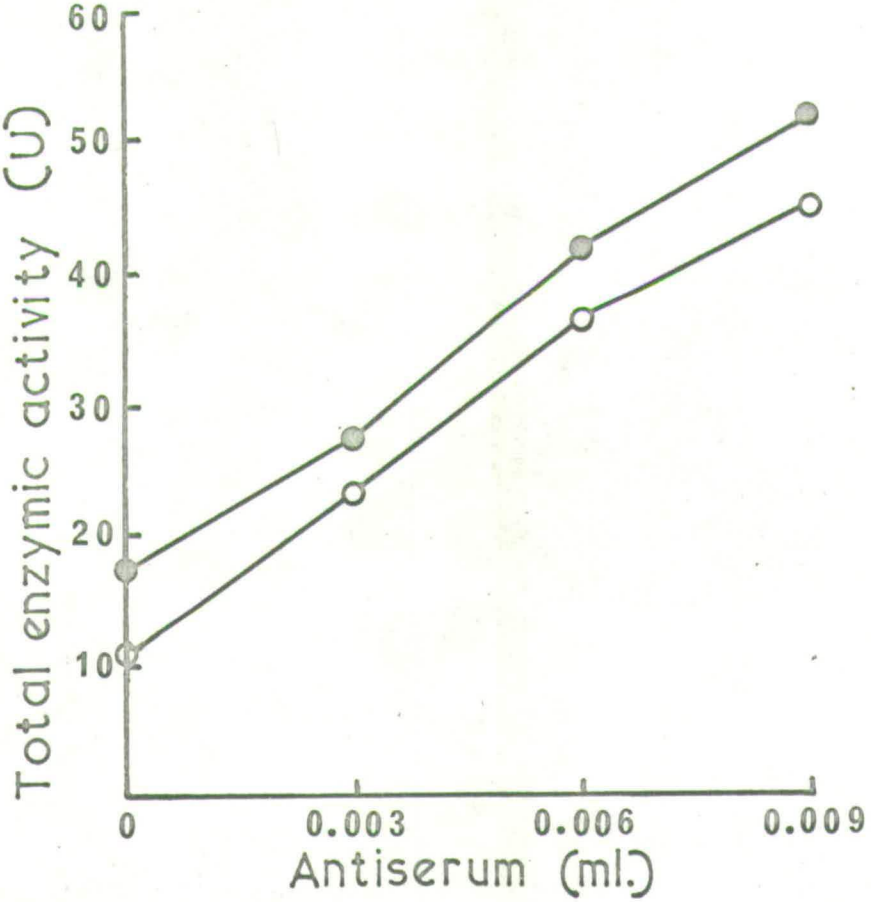


Fig. 4. The antigenic reaction of the extracellular penicillinase from the mesoconstitutive mutant K19 (O) compared with reaction of unmutated A-type penicillinase from strain PC1 (●). The antiserum was prepared against purified A-type enzyme (Richmond, 1963). The experimental details are given in the text.

genes are equally expressed. These tests were carried out in host strain 8325. As tests of this kind have been used extensively in this thesis, mainly in strain 147, and use has been made of magno-constitutive strains to test for the presence of an $\underline{i}^+$ region, the behaviour of a magno-constitutive/magno-inducible ($\underline{i}^-/\underline{i}^+$) diploid in strain 147 is described again below.

The magno-constitutive mutant, K3, carrying the linked markers $\underline{Hg}^S\ \underline{Em}^R$ (by recombination) was used as the source of transducing phage and the strain 147: $\underline{\beta}\ \underline{i}^+\ \underline{p}_C^+\ \underline{Hg}^R\ \underline{Em}^S$ as recipient. Transductants were selected with erythromycin and diploid clones isolated, purified and confirmed as described in Section II.12. The behaviour of this diploid, together with the parent strains and the segregants are shown in Table 4a.

In a similar manner the analogous diploid was constructed in which the magno-constitutive mutation is carried on the $\underline{\beta}$ plasmid, by using 147C223 (147: $\underline{\beta}\ \underline{i}^-\ \underline{p}_C^+\ \underline{Hg}^R\ \underline{Em}^S$) as recipient and P_o (8325: $\underline{\alpha}\ \underline{i}^+\ \underline{p}_A^+\ \underline{Hg}^S$ --- $\underline{\chi}\ \underline{Em}^R$) as donor, and the properties of this diploid is shown in Table 4b.

The properties of these diploids may be summarised as follows:

(a) The total amount of penicillinase synthesised by the diploids when fully induced is similar to that of the normal haploid strain when induced, or to the magno-constitutive parent.

(b) The activity of the uninduced diploid is 5 - 12 U/mg. dry wt. of bacteria, compared with about 3 - 5 U/mg. for the $\underline{\alpha}\ \underline{i}^+\ \underline{p}_A^+$ plasmid and 1 - 3 U/mg. for the $\underline{\beta}\ \underline{i}^+\ \underline{p}_C^+$ plasmid in the haploid state.

Table 4. The properties of diploid strains obtained from two experiments involving magno-constitutive mutants and wild type inducible strains.

In (a) the diploid was constructed by transducing the magno-inducible strain 147i (147: $\underline{\beta}\ \underline{i}^{+}\ \underline{p_C}^{+}\ \underline{Hg}^{R}\ \underline{Em}^{S}$) with phage from the erythromycin-resistant version of the magno-constitutive mutant K3 (8325: $\underline{\alpha}(K3)\underline{p_A}^{+}\ \underline{Hg}^{S}$ --- $\underline{\gamma}\ \underline{Em}^{R}$) (Table 3). In (b) the diploid was constructed by transducing the magno-constitutive strain 147C223 (147: $\underline{\beta}\ \underline{i}^{-}\ \underline{p_C}^{+}\ \underline{Hg}^{R}\ \underline{Em}^{S}$) with phage from the magno-inducible strain $P_o\ \underline{Hg}^{S}\ \underline{Em}^{R}$ (8325: $\underline{\alpha}\ \underline{i}^{+}\ \underline{p_A}^{+}\ \underline{Hg}^{S}$ --- $\underline{\gamma}\ \underline{Em}^{R}$). The properties of the parental strains and the haploid segregants of the diploids are also shown.

Strain	Penicillinase activity (U/mg.) Uninduced	Induced	Induction ratio
(a) Parental strains			
K3 (donor)*	354	365	1·0
147i (recipient)	1	159	159
Diploid*	8	279	35
Segregants			
147:K3≠	374	373	1·0
147i	1	136	136
(b) Parental strains			
P_o (donor)	3	273	91
147C223 (recipient)	260	228	0·9
Diploid	9	294	33
Segregants			
147:P_o≠	3	147	49
147C223	183	171	0·9

* Mean of 2 values in each case.

≠ 147:K3 and 147:P_o have the characteristics of the $\underline{\alpha}$-plasmids from K3 and P_o respectively in host strain 147.

These properties in 147 cytoplasm are like those of the equivalent diploids constructed in strain 8325 (Richmond, 1965b & c) in showing that the expression of the region for full inducibility ($\underline{i}^+$), acting *trans* in a diploid is dominant over that for magno-constitutivity ($\underline{i}^-$) with repression of its basal enzyme activity. It has been shown elsewhere (Richmond, 1965b & personal communication) that with induction the limit to the expression of the genes on both plasmids is removed, allowing equal expression of both structural (p) genes. The dominant effect of $\underline{i}^+$ over $\underline{i}^-$ is independent of the plasmid of origin of the $\underline{i}^+$, $\underline{\alpha}$ or $\underline{\beta}$, and whether it originated in the donor or recipient in the transduction. Results of this kind may be interpreted as being due to the intact $\underline{i}^+$ allele determining a cytoplasmic product which is active in repressing gene expression while the magno-constitutive allele does not make this regulator gene product, or makes one that is inactive and does not compete with the ability of the $\underline{i}^+$ gene product to act *trans* in the diploid. Magno-constitutive mutants which behave in this way in diploids are designated $\underline{i}^-$. The cytoplasmic repressor might be a protein molecule as for the *lac* operon of *E. coli* (Gilbert & Muller-Hill, 1966) and the λ phage of *E. coli* (Ptashne, 1967), but it should be noted that there are other possible mechanisms for *trans*-dominant repression which might apply. For example, were the i-gene to be transcribed in the same RNA molecule as the other genes of the operon, and the messenger RNA translated at only a limited number of sites, which in the absence of inducer, become blocked by the $\underline{i}^+$ product, the expression of the other genes

both *cis* and *trans* to i^+ would be prevented. The i^- product would need to be released by these sites so rapidly that it could not interfere with the i^+ blockage which is itself removed in the presence of inducer molecules. A scheme of this type cannot easily explain the specificity of induction in terms of what is at present believed to be the mechanism of protein translation (Lengyel, 1966).

3. SEMI-CONSTITUTIVE MUTANTS

The magno-constitutive strains discussed in the previous section have fully derepressed basal levels of enzyme and no further response to inducer; in fact, the highest induction ratio of the mutants arbitrarily grouped under this heading is 1·2 (see Table 3). Another class of constitutive mutants, the semi-constitutive, has a high basal amount of enzyme which is less than the normal maximum induced level (300 U/mg.), but increased to this value on induction, and hence has an induction ratio greater than the magno-constitutive but less than the wild type strain (defined as 30). Mutants of this kind have been examined by Richmond (1965c), and appear to be partial i^- mutants, repressed by an i^+ allele *trans*, but making a regulator gene product which has not entirely lost the ability to block structural gene expression, and which retains unimpaired its normal ability to interact with the inducer. No further mutants of this type were examined here.

4. BASO-CONSTITUTIVE AND SEMI-INDUCIBLE MUTANTS

Mutants synthesising basal levels of enzyme similar to the wild type strain (5 - 10 U/mg.) and that are not inducible are called baso-constitutive. Mutants of a similar uninduced activity which are partially inducible to a level below the normal maximum (300 U/mg.) are called semi-inducible. Both of these phenotypes arise from a regulator gene mutation in that it lies outside the penicillinase structural gene, so that the enzyme is unchanged. In both cases the strains appear to be $\underline{i}^+$ as judged by their ability to repress the magno-constitutive $\underline{i}^-$ phenotype in heterogenotes; and this other regulator mutation is recessive to the unmutated allele such that, in the diploid strains, the structural gene *cis* to the mutation is induced to the normal diploid amount (Richmond, 1967b, & personal communication). The existence of mutants of this type is evidence for a locus determining a regulatory function affecting the induction response. This locus is independently mutable to the locus for the repressor function, and is complemented independently to it in heterogenotes. The product determined by this locus may be totally inactive as in the baso-constitutive mutants, or the product may not be completely inactive but, rather, its function may be quantitatively altered as in the semi-inducible mutants. Both types of mutant make a repressor product unimpaired in its ability to block the structural gene expression. Since these classes of constitutive mutation were being examined elsewhere, none were studied further here.

5. MESO-CONSTITUTIVE MUTANTS

(a) Description of mutant, K19

Mutants classed as meso-constitutive were those with basal enzyme levels above the wild type (10 U/mg.) and induced levels below the normal maximum (300 U/mg.) such that the induction ratio was less than 5 (see Section I). Five mutants of this phenotype were isolated among the haploid mutants described above (Section III.1.). As the genetic basis for this penicillinase phenotype in S. aureus was not known, one of the mutants (K19) was chosen for study. The erythromycin-resistant recombinant of the mutant plasmid had a penicillinase activity of 40 - 50 U/mg. uninduced and 30 - 60 U/mg. after induction, with an induction ratio not significantly different from 1 (See Table 5). This mutant appeared to make antigenically unaltered enzyme, and hence the mutation probably lies in a region outside the locus for p_A^+ (see Section III.1).

(b) Induction of K19

The rate of penicillinase synthesis by mutant K19 was not increased by induction under standard conditions. One possible reason for the absence of induction might be an alteration in the affinity for the inducer of one site concerned in the induction process. If this were so, then an increase in the concentration of inducer, or the use of another inducer compound might lead to effective enzyme induction.

To test this possibility, cultures for induction were set up in the usual way except that methicillin was added to give final

Table 5. The properties of the erythromycin-resistant version of the meso-constitutive mutant K19 (Table 1).
In (a) the properties are shown of the erythromycin-resistant recombinant (8325: α(K19) p_A^+ Hg^S --- γ Em^R) of strain K19.
In (b) the properties are shown of transductant clones obtained by transducing the plasmid-negative strain 147 N with phage from the erythromycin-resistant version of strain K19 (a).
In (c) the properties are shown of the meso-constitutive haploid segregants (K19) obtained from the diploids constructed by transducing strains 147i and 147C223 with phage from the erythromycin-resistant version of strain K19 (a).

Strain	Penicillinase activity (U/mg.) Uninduced	Induced	Induction ratio
(a) K19	54	57	1·1
	41	37	0·9
	51	34	0·7
Mean	49	43	0·9
(b) Transductants (K19) of strain 147 N	51	26	0·5
	32	31	1·0
(c) Segregants (K19) in strain 147	51	39	0·8
	38	44	1·2
	27	40	1·5
	40	54	1·4
	34	48	1·4
	35	50	1·4
	29	28	1·0
	37	19	0·5
	30	24	0·8
Mean	36 ± 7	39 ± 12	1·1

concentrations of 0·1 to 2·0 μg./ml., or cephalosporin C to give 1 to 200 μg./ml. The wild type magno-inducible strain, P_o, was included for comparison. As shown by the results in Table 6, K19 was not induced by the increased concentrations of either inducer. As also shown in Table 6, the increase in concentration of both compounds was limited by the inhibition of growth that they caused.

(c) Complementation tests.

The plasmid from strain K19 was tested in heterodiploids with both the wild type i^+ allele and the magno-constitutive i^- allele in the trans position for the effect on enzyme repression and for restoration of inducibility.

The plasmid from strain K19 Hg^S Em^R was transduced into 147i, the 147 strain carrying β i^+ p_C^+ Hg^R Em^S, and diploid colonies were detected by their double resistance for Em and Hg, and confirmed by the segregation of haploid clones with the resistance and enzyme characteristics of the parent strains. The properties of two isolates of this diploid are shown in Table 7. The two clones were constructed independantly from separate phage stocks and bacterial cultures. In the presence of the i^+ allele the p_A gene is repressed, and the total enzyme level is characteristic of the i^+/i^- diploid (see Table 4).

After induction, the total amount of enzyme made by the diploid is similar to that of 147i alone. The supernatant enzyme was analysed quantitatively with antiserum and the content of A-type penicillinase calculated. Values for this of 82 and 95% of the

Table 6. The induction of penicillinase in the meso-constitutive mutant K19 and in the inducible wild type strain P_o (8325: $\underline{a}\ \underline{i}^+\ \underline{p}_A^+$); the penicillinase activity and extent of bacterial growth at the end of the induction period for several concentrations of methicillin or cephalosporin C.

Inducer Concentration (µg./ml.)	Penicillinase activity (U/mg.)				Bacterial density (mg. dry wt./ml.)			
	Inducer compound				Inducer compound			
	Methicillin		Cephalosporin C		Methicillin		Cephalosporin C	
	Strain		Strain		Strain		Strain	
	P_o	K19	P_o	K19	P_o	K19	P_o	K19
0	5	48	2	55	3·5	1·9	1·2	1·6
0·1	21	54			2·9	1·9		
0·2	76	52			2·7	1·9		
0·5	101	58			2·3	1·7		
1·0	113	58	12	70	0·9	0·6	1·0	1·7
2·0	160	33	19	57	0·3	0·2	1·1	1·6
5			60	51			0·8	1·7
10			78	54			0·9	1·7
20			132	61			0·4	1·5
50			101	67			0·1	1·2
100			-	65			<0·1	0·3
200			-	68			<0·1	0·2

Table 7. The properties of two diploid strains obtained, in independent experiments, by transducing strain 147i (147: $\underline{\beta}\ \underline{i}^{+}\ \underline{p}_{C}^{+}\ \underline{Hg}^{R}\ \underline{Em}^{S}$) with phage from the erythromycin-resistant version of the meso-constitutive strain K19 (8325: $\underline{\alpha}$ (K19) $\underline{p}_{A}^{+}\ \underline{Hg}^{S}$ --- $\underline{\gamma}\ \underline{Em}^{R}$). The properties of the parental strains and of the haploid segregants from the diploids are also shown.

Strain	Total penicillinase activity (U/mg.)		Extracellular penicillinase		
			% of total activity		Antigenic type
	Uninduced	Induced	Uninduced	Induced	
Parental strains					
K19 (donor)	32	31	44		A
147i (recipient)	1	80		6	C
Diploid (1)*	9	111	10 - 67	31 - 35	A + C
Segregants from diploid (1)					
147:K19*≠	39	41	27 - 29		A
147i*	2	88		7 - 16	C
Diploid (2) ≠	8	234	17	25	A + C
Segregants from diploid (2)					
147:K19≠ ≠	35	49			A
147i≠	1	114			C

* Mean of 4 values in each case.

≠ 147:K19 has the characteristics of the $\underline{\alpha}$-plasmid from strain K19 in host strain 147.

≠ Mean of 2 values in each case.

total supernatant enzyme activity were obtained, and these values are consistent with those expected when both the structural genes in the diploid are equally expressed. The calculation showed that the total A-penicillinase made by this diploid is about 5 U/mg. uninduced and 60 - 80 U/mg. induced. The induction ratio is therefore about 16, a value consistent with there being half the haploid value of expression for each structural gene in the diploid. The evidence of this diploid shows that K19 behaves like a $\underline{i}^{=}$ mutant (see magno- and semi-constitutive mutants, Sections II.2 and II.3) in that its rate of penicillinase synthesis is sensitive to repression in the presence of a normal $\underline{i}^{+}$ allele, and also that the function altered to cause loss of inducibility is restored in the presence of the wild genotype.

Similarly, diploids were constructed by transduction of K19 $\underline{Hg}^{S}\ \underline{Em}^{R}$ plasmid into 147C223, carrying the $\underline{\beta}\ \underline{i}^{-}\ \underline{p_C}^{+}\ \underline{Hg}^{R}\ \underline{Em}^{S}$ plasmid. In this diploid the uninduced level of enzyme was similar to that of K19, i.e. the expression of the $\underline{p_C}^{+}$ gene has been repressed from the magno-constitutive level to 60 U/mg., and the total induced level is similar to 147C223, as shown in Table 8. The supernatant enzyme was analysed as described above, and the total A-type penicillinase made by the diploid was calculated to be about 20 U/mg. uninduced and 160 - 270 U/mg. after induction; the induction ratio is therefore about 10, and possibly as high as 14, for this enzyme type. The behaviour of this diploid suggests that a product determined by the K19 plasmid retains some repressor activity for both the *cis* and *trans* structural genes, and also that full inducibility is restored to both these genes by a function

Table 8. The properties of two diploid strains obtained, in independent experiments, by transducing strain 147C223 (147: $\underline{\beta}$ $\underline{i}^-$ $\underline{p}_C^+$ $\underline{Hg}^R$ $\underline{Em}^S$) with phage from the erythromycin-resistant version of the meso-constitutive strain K19 (8325: $\underline{\alpha}$ (K19) $\underline{p}_A^+$ $\underline{Hg}^S$ --- $\underline{\gamma}$ $\underline{Em}^R$). The properties of the parental strains and of the haploid segregants from the diploids are also shown.

Strain	Total penicillinase activity (U/mg.)*		Extracellular penicillinase*		
			% of total activity		Antigenic type
	Uninduced	Induced	Uninduced	Induced	
Parental strains					
K19 (donor)	51	34	38		A
147C223 (recipient)	188	170	5		C
Diploid (1)≠	60	205	21 - 37	41 - 62	A + C
Segregants from diploid (1)					
147:K19≠#	40	34	35	45	A
147C223≠	162	167	4	6	C
Diploid (2)	55	293	24 - 26	35 - 37	A + C
Segregants from diploid (2)					
147:K19#	34	22	29 - 50		A
147C223	197	219	2		C

* Unless otherwise stated the results are the mean of 2 values in each case.
≠ Mean of 9 values in each case.
≠ Mean of 3 values in each case.
147:K19 has the characteristics of the $\underline{\alpha}$-plasmid from strain K19 in the host strain 147.

determined by a genome which has a magno-constitutive mutation in the i-gene.

(d) Transduction of K19 to a new host cell

The effect of the bacterial genetic background on the meso-constitutive phenotype was tested by transducing the plasmid from K19 Hg^S Em^R into the different, plasmid-less host, 147 N. Nine erythromycin-resistant transductants were obtained and found to be similar in properties to the original K19 strain when screened by penicillin-iodine reagent on CY agar plates. Two clones were assayed quantitatively by the Perret method and the enzyme found still not to be inducible. Their basal penicillinase levels were about 40 U/mg., and their induced levels were about 30 U/mg. These values are similar (within 2 standard deviations of the mean) to those obtained for the segregants of the K19 plasmid arising in strain 147 from the diploids used in the complementation tests above (see Table 5). Similarly, when K19 was transduced into P16, the plasmid negative derivative of host strain, 8325, all of the 280 Em^R colonies selected had basal activities similar to K19 as measured by the penicillin-iodine reaction. Therefore the meso-constitutive phenotype is due to a plasmid-linked mutation, and is not dependent on the genetic background of the host strain.

(e) Recombination between "repressor" and "inducer" cistrons

In the meso-constitutive mutant, K19, two separate regulator functions appear to have been partially or totally lost; one is some kind of repressor function in that the basal level is high and the other is some inducer function in that there is no induction

response. This situation could be the result either of two separate point mutations, one affecting each function, or of a single mutational event encompassing both. As it has been shown that the functions are independently restored by complementation, they may actually be determined by separate cistrons. In the following discussion the gene for the induction property is called $\underline{i}_N$ and for the repression property $\underline{i}_R$. If two separate mutations have occurred it should be possible to recombine in the region between them. To test this possibility, K19 was crossed with the unmutated plasmid of P_o, ($\underline{a}$ $\underline{i}_R^+$ $\underline{i}_N^+$ $\underline{p}_A^+$). If two mutations are correct the possible progeny from such a cross together with their predicted phenotype are

	Genotype	Predicted penicillinase phenotype		
		Uninduced activity (U/mg.)	Induced activity (U/mg.)	
Parental P_o	$\underline{i}_R^+$ $\underline{i}_N^+$ $\underline{p}_A^+$	5	300	(i)
Parental K19	$\underline{i}_R^-$ $\underline{i}_N^-$ $\underline{p}_A^+$	50	50	(ii)
Recombinant	$\underline{i}_R^+$ $\underline{i}_N^-$ $\underline{p}_A^+$	5	5	(iii)
Recombinant	$\underline{i}_R^-$ $\underline{i}_N^+$ $\underline{p}_A^+$	50	300	(iv)

The baso-constitutive phenotype (iii) should be easily distinguishable from the other phenotypes expected from this cross under induction conditions, and so was sought in an attempt to find evidence for recombination. In construction experiments on CY agar containing an inducer this phenotype could be detected in a mixture containing also K19, P_o and a semi-constitutive mutant, when present as 1% of the total count.

In order to carry out the cross, transducing phage from P_o (8325: $\underline{\alpha}$ $\underline{i}^+$ $\underline{p}_A^+$ --- $\underline{\gamma}$ $\underline{Em}^R$) was absorbed to K19 $\underline{Em}^S$ for 50 min. at 37°, centrifuged, resuspended in fresh medium, and incubated for a further 4 or 24 hr., since in preliminary experiments where the bacteria were plated immediately after phage absorption, they gave rise to mixed colonies, indicating that the plasmids had not had time to segregate. Transductants were detected by selecting for the independent marker, $\underline{Em}^R$ introduced on the transduced plasmid, the bacteria being plated on to CY agar containing erythromycin and cephalosporin C. Colonies of uncertain phenotype were re-plated and retested.

No baso-constitutive colonies were obtained from among 8,000 $\underline{Em}^R$ colonies obtained by plating after 4 hr. incubation, nor were any found among 114,000 $\underline{Em}^R$ transductants plated after overnight growth. This result strongly suggests that the mutational changes giving rise to the meso-constitutive phenotype are inseparable by recombination.

(f) Conclusions

The mutant K19 differs from wild phenotype for penicillinase synthesis in two ways: (i) it synthesises derepressed amounts of penicillinase; and (ii) it is not inducible to increased amounts. The enzyme synthesised is not a mutein, and the genetic change (or changes) giving rise to this phenotype is determined by the plasmid that carries the penicillinase structural gene ($\underline{p}_A^+$) and not by an unlinked suppressor mutation. The phenotype can therefore be ascribed to mutation in a regulatory region (or regions); the

information about the location of the alteration and the functions affected are summarised below.

(i) Repression

The increased basal level of enzyme made by K19 might be due to mutation of an operator (O) region or of the regulatory (i) region previously described (see Sections II.2 and 3). In heterodiploids with an i^+ allele in the *trans* position, K19 behaves like a derepressed constitutive mutant (i^-). The possibility of a mutation in O is excluded by this sensitivity to the i^+ regulatory gene product. However, the i^- mutation in strain K19 does not lead to total derepression of enzyme synthesis which instead corresponds to the derepression of a partial i^- (semi-constitutive) mutant. Moreover, in heterodiploids with a magno-constitutive i^- allele *trans* to the K19 regions, the enzyme production of the magno-constitutive mutant is repressed, and a basal level similar to that of K19 is produced by the diploid. Thus it would seem that K19, like the semi-constitutive mutants, is making a repressor which is so damaged as to be partially but not completely inactive. If this is the case, the lack of inducibility of K19 must be due to a second genetic change.

(ii) Induction

The loss of inducibility of the mutant K19 might be due either to a mutation in a promoter (pr) region, or in another region affecting regulation of penicillinase. The limit to the expression of a structural gene *cis* to a promoter mutation (pr^-) is retained in a diploid with the wild type allele *trans*. In complementation

tests with either the unmutated β-plasmid carrying an i^+ gene or with a β-plasmid carrying an i^- allele in the *trans* position, the inducibility of the p_A^+ gene of K19 was restored. Therefore, K19 does not have a pr^- mutation. The complementation evidence also suggests that the mutation affecting inducibility lies in a different cistron to the one involved in determining the level of repression. Full inducibility of the total enzyme was expressed by both types of diploid, and, for the structural gene of K19, was restored by some function determined by the other plasmid, irrespective of the repressor activity determined by this plasmid. Thus induction to the wild type diploid level was obtained with K19 even when there was a magno-constitutive i^- mutation on the *trans* plasmid determining the induction function, and the repressed level of the complementing diploid was determined by the K19 allele. The alternative hypothesis is intracistronic complementation, with both regulatory functions of repression and inducibility determined by the same cistron, but the arguments against one cistron and in favour of two are, firstly, that the level of induction achieved was similar to that of diploids carrying only wild type alleles for inducibility, although this property can be quantitatively graded as evidenced by the existence of the semi-inducible mutants; and, secondly that the same degree of inducibility has been obtained in complementation tests with the β i^- plasmid for all the other mutants of the induction response (baso-constitutive and semi-inducible mutants) that have been tested (Richmond, 1967b). Therefore it seems probable that two cistrons are involved, one determining the repressed enzyme level and the

other the induction response; these two genes are referred to as $\underline{i}_R$ and $\underline{i}_N$ respectively.

With the mutant K19 the loss of inducibility appears to be total in that no increase in penicillinase production occurred with this strain. This loss applied with two different effector compounds and induction could not be forced by increasing the concentration of either inducer. This behaviour makes it unlikely that there has merely been a change in the affinity for the effector molecule of a site involved in the induction response or that an enzymic reaction is involved. This point is discussed at greater length below, together with some possible functions determined by the $\underline{i}_N$ regulatory region (see general discussion, Section VI).

Although the complementation data for mutant K19 suggested that two regulatory loci, $\underline{i}_N$ and $\underline{i}_R$, have been changed by mutation, the evidence of the recombination experiments was that the alterations can be attributed to a single mutational event rather than to two separate ones at separable sites. In an attempt to effect recombination in the region between two separate mutations, hypothesised as one affecting each of the two loci concerned, no such recombinants were obtained in over 120,000 erythromycin-resistant transductants.

IV. THE SEARCH FOR OPERATOR-CONSTITUTIVE MUTANTS

1. OPERATOR-CONSTITUTIVE MUTANTS

In the lac system of E. coli, a class of semi-constitutive mutants have been isolated with properties different from those of the i^- mutants described above (Section III.3.). The constitutivity of these mutants is expressed in the presence of an i^+ gene, but only by the structural gene cis to the mutation. The insensitivity to the i^+ product led to the suggestion that the mutation lies in the region responsible for recognising this product and mediating its action of limiting structural gene expression. (Jacob & Monod, 1961). This region was called the operator (O) region. Originally it was further defined by a second class of mutant, called operator-zero (O^o), limiting structural gene expression, but these have since been shown to map outside O and be unrelated to its function (Beckwith, 1964b). Thus the derepressed mutants, designated operator-constitutive (O^c), are at the present the only evidence for the existence of the operator region (Beckwith, 1967).

No cis-dominant operator-constitutive mutation has yet been found for penicillinase in S. aureus, but, because of the importance of this type of mutant in identifying an operator region in a regulatory system, an attempt was made to isolate such a mutant, based on the methods used by Jacob, Ullman & Monod (1964) for isolation for the lac operon. They found that O^c mutants arose spontaneously or after EMS or X-ray mutagenesis, but neither after u.v. nor 2-aminopurine treatment. To facilitate selection, these authors used a strain that was diploid for the i region, either i^+

or, preferably, $\underline{i}^s$. The $\underline{i}^s$ mutant (super-repressed) makes a functional repressor which no longer responds to inducer, and this non-inducibility is trans-dominant (Willson, Perrin, Cohn, Jacob & Monod, 1964). Theoretically, the only class of mutants which can synthesise enzyme at greater than basal rate is that which can escape the dominant repressive action of both $\underline{i}^s$ regions. With staphylococcal penicillinase no $\underline{i}^s$ mutants were available, so the unmutated $\underline{i}^+$ allele was used instead. Treatment of an $\underline{i}^+/\underline{i}^+$ diploid should yield mutant strains expressing derepressed penicillinase levels only if one component of the diploid is a constitutive of the $\underline{o}^c$ type.

2. SEARCH PROCEDURE

The unmutated $\underline{\alpha}$-plasmid from 8325: $\underline{\alpha}\ \underline{i}^+\ (\underline{o}^+)\ \underline{p}_A^+\ \underline{Hg}^S$ --- $\underline{\gamma}\ \underline{Em}^R$ was transduced into 147 carrying the unmutated $\underline{\beta}$-plasmid, i.e. $\underline{\beta}\ \underline{i}^+\ \underline{p}_C^+\ \underline{Hg}^R\ \underline{Em}^S$, and the transductants selected with erythromycin. Most transductants proved to be diploid and were fully inducible and resistant to both erythromycin and mercury salts. The diploid state could be shown because they segregated both parental phenotypes (see Table 9). The $\underline{i}^+/\underline{i}^+$ diploid obtained in this way was treated with EMS or with X-rays and plated on CY agar to allow unselected screening of the colonies for the amount of penicillinase produced. Clones were picked for further examination if the level of enzyme expression was greater than that of the $\underline{i}^+/\underline{i}^+$ diploid which formed most of the colonies on the plate. The

Table 9. The properties of the diploid strain obtained by transducing strain 147i (147: $\underline{\beta}\ \underline{i}^{+}\ \underline{p}_{C}^{+}\ \underline{Hg}^{R}\ \underline{Em}^{S}$) with phage from strain $P_o\ \underline{Hg}^{S}\ \underline{Em}^{R}$ (8325: $\underline{\alpha}\ \underline{i}^{+}\ \underline{p}_{A}^{+}\ \underline{Hg}^{S}$ --- $\underline{\gamma}\ \underline{Em}^{R}$). The properties of the haploid segregants obtained from the diploid and the parental strains are also shown.

Strain	Total penicillinase activity (U/mg.)		Extracellular penicillinase activity from the induced culture	
	Uninduced	Induced	% of total activity	Antigenic type
Parental strains				
P_o (donor)	4	167	53	A
147i (recipient)	1	97	12	C
Diploid	1	119	44	A + C
Segregants				
147:P_o*	3	180	70	A
147i	1	136	17	C

* 147:P_o has the characteristics of the $\underline{\alpha}$-plasmid from strain P_o in host strain 147.

diploid nature of the clones isolated was confirmed by showing that they were still resistant to both erythromycin (a characteristic of the α --- γ-plasmid) and to mercury salts (a characteristic of the β-plasmid), and that they segregated haploid clones which were resistant to one of these agents, but not to both.

3. MUTANT DIPLOIDS OBTAINED WITH EMS

A total of 61,000 colonies of the diploid 147: α i^+ p_A^+ Hg^S --- γ Em^R/β i^+ p_C^+ Hg^R Em^S were screened after EMS treatment in 3 separate experiments. Out of 130 clones picked because they seemed to have a higher level of penicillinase synthesis than the parent strain, 18 had a basal level significantly greater than that of the diploid parent when examined quantitatively and were still doubly resistant, indicating the presence of both parental markers, Em^R and Hg^R. Of these 18 isolates, 10 had basal enzyme levels between 5 and 13 U/mg. and segregated to give both a magno- or semi-constitutive segregant and a magno-inducible one. These 10 mutant clones were therefore i^+/i^- diploids in which the raised basal level of enzyme was due to the slightly higher uninduced level found in such diploids when compared with the parental i^+/i^+ diploid (see Table 4, Section III.2 and Table 9, Section IV.2). In 5 of the mutants the constitutive mutation was on the α-plasmid and in the other 5 it was carried by the β-plasmid.

A further 5 isolates were found in practice to be i^+/i^- diploids in which the uninduced level of penicillinase synthesis was

greater than that normally associated with such diploids. Examination of cultures of these isolates showed that frequently as much as 50% of the culture was in the form of constitutive haploid segregants. Although no attempt was made to discover whether this type of mutant arose by single or multi-site mutation, its phenotypic properties could be accounted for if mutation, as well as creating the constitutive state for one component of the diploid, has conferred instability on the other.

Three mutants were found which made penicillinase constitutively and stably retained both resistance markers. These diploids segregated to a derepressed constitutive clone carrying one of the resistance markers, and a clone which carried the second resistance marker and which appeared to make little or no penicillinase as expected for an $\underline{i}^+$ genotype. Since the constitutive segregants in diploids of this type have the properties expected of operator constitutive mutants, one of them (E14) was examined in greater detail.

Diploid mutant E14

The mutant diploid, E14, obtained by EMS treatment of the $\underline{i}^+/\underline{i}^+$ diploid, had an uninduced penicillinase level of about 150 U/mg. but frequently was found to assay at values less than this (70 - 90 U/mg.) because of the proportion of negative cells that rapidly accumulated in cultures. Less than 5% of the total penicillinase of the diploid was released from the bacteria, whether in the presence or absence of inducer, and the exoenzyme activity was not increased by antiserum to A-type penicillinase. The mutant is,

therefore, unlike the parent diploid which liberates about 25% of the total enzyme into the medium, and this exoenzyme is stimulated by antiserum; instead the mutant resembles strains synthesising only C-penicillinase.

Two segregants were obtained from this diploid. The first segregant from E14 was $\underline{Hg}^S$, $\underline{Em}^R$, and $\underline{Cd}^R$, like the original $\underline{\alpha}$ --- $\underline{\gamma}$-plasmid of the $\underline{i}^+/\underline{i}^+$ diploid, but made no detectable penicillinase whether inducer was present or not. The lower limit of enzyme detectable by the Perret assay is 0·5 U/mg.. When this plasmid was transduced into the stock $\underline{i}^-$ strain, 1470223, no repression of its penicillinase synthesis was observed in clones plated on CY agar and stained with the penicillin-iodine reagent. Therefore this segregant had lost all measurable activity for both penicillinase loci, $\underline{i}$ and $\underline{p}$, and was concluded to have arisen from the $\underline{\alpha}$-plasmid of the original $\underline{i}^+/\underline{i}^+$ diploid probably by deletion of both loci.

The second segregant, the putative $\underline{\beta}$-plasmid segregant, carried the appropriate resistance markers, $\underline{Hg}^R$ $\underline{Em}^S$, and assayed for penicillinase at about 150 U/mg. of which less than 5% was extracellular. This behaviour is consistent with only C-type penicillinase being synthesised by the segregant. This segregant was tested for the repressibility of its enzyme synthesis by using it as recipient for transduction of an $\underline{i}^+$ allele on the $\underline{\alpha}$-plasmid from P_o $\underline{Hg}^S$ $\underline{Em}^R$. Most doubly resistant clones from this transduction appeared to be normal stable diploids segregating both parental types, and with a repressed basal level of penicillinase

similar to that of the $\underline{i}^+/\underline{i}^-$ diploid discussed above. An example of these diploids is shown in Table 10. However, 5 - 10% of doubly resistant clones made a basal level of 130 U/mg.. From these bacteria, both induced and uninduced, less than 10% of the enzyme was extracellular and no stimulation with anti-A serum occurred, nor were any singly resistant segregants found among nearly 2,000 colonies of this type of supposed diploid. In order to test whether the two plasmids could be recovered from this doubly resistant strain, transducing phage was prepared from it and used to infect the plasmid-negative host strain, 147 N. As both of the parental plasmids carried the marker for cadmium resistance, transductants were selected on CY agar containing cadmium ions. All the markers, $\underline{Cd}^R$, $\underline{Hg}^R$, $\underline{Em}^R$, and high penicillinase synthesis, were transduced together in 450 colonies isolated and this is consistent with them being on a single linkage group. Thus the second segregant from E14 makes derepressed levels of penicillinase and is derived from the $\underline{\beta}$-plasmid by mutation of the $\underline{i}$-gene. Mutation has also altered either the plasmid or the host cell with the consequence of recombination being more frequent than usual between this $\underline{\beta}$-plasmid and an $\underline{\alpha}$-plasmid introduced into the same cell.

The original mutant diploid, E14, examined here was, therefore, genetically $\underline{\alpha}\ (\underline{i}\ \underline{p})^{del.}\ \underline{Cd}^R\ \underline{Hg}^S$ --- $\gamma\ \underline{Em}^R/\underline{\beta}\ \underline{i}^-\ \underline{p}_C^+\ \underline{Cd}^R\ \underline{Hg}^R\ \underline{Em}^S$ and the high level of enzyme synthesised was because there was no $\underline{i}^+$ allele in the *trans* position not because of an $\underline{O}^c$ mutation.

Table 10. The properties of the diploid strain and of a recombinant strain obtained when phage from strain P_o (8325: $\underline{a}$ $\underline{i}^+$ $\underline{p}_A^+$ $\underline{Hg}^S$ --- $\underline{\gamma}$ $\underline{Em}^R$) was used to transduce the constitutive haploid segregant (strain 147 carrying the $\underline{\beta}$ (E14) $\underline{Hg}^R$ $\underline{Em}^S$-plasmid) of the diploid mutant E14. The properties of the parental strains and of the haploid segregants of the diploid are also shown. Strains similar to the recombinant strain occurred at a frequency of 5-10% of the diploid type.

Strain	Penicillinase activity (U/mg.)		Induction ratio
	Uninduced	Induced	
Parental strains			
P_o (donor)	5	167	33
$\underline{\beta}$-plasmid segregant from E14* (recipient)	156	263	1·7
Diploid	5	316	63
Segregants			
147:P_o ≠	4	128	32
E14 $\underline{\beta}$-plasmid segregant	233	352	1·5
Recombinant*	133	253	1·9

* Mean of 2 values in each case.

≠ 147:P_o has the characteristics of the $\underline{a}$-plasmid from strain P_o in the host strain 147.

4. MUTANT DIPLOIDS OBTAINED WITH X-RAYS

For X-ray mutation of the i^+/i^+ diploid, four radiation doses of 23000, 37000, 55000 and 74000 roentgen (corresponding to killing doses of 46, 62, 94 and 98% of the initial viable count) were used. In these experiments a total of 110,000 colonies were scanned, but, of the 84 clones picked for preliminary investigation, only three still carried both the resistance markers and had a basal level of enzyme above that of the original diploid. One isolate behaved as though it were an i^+/i^- diploid in making uninduced penicillinase at less than 10 U/mg. and segregating to a normal, repressed, α-plasmid haploid, and also to a β-plasmid derivative with magno- or high semi-constitutive phenotype. The other two mutant isolates made large amounts of penicillinase similar to the amount synthesised by one of their haploid segregants, while the other segregant had little or no enzyme activity. One of these diploids, X35, was examined in detail.

Diploid mutant X35

The diploid mutant, X35, constitutively made penicillinase at about 160 U/mg., and segregated one haploid carrying the α --- γ-plasmid markers, Hg^S, Em^R, and making 150 U/mg.. The effect of a *trans* i^+ allele on this segregant was tested by transduction of the plasmid into strain 147i which carries β i^+ p_C^+ Hg^R Em^S. The diploid colony formed had repressed basal enzyme levels and segregated clones of both parental types as shown in Table 11. The constitutivity of this segregant is therefore due to a mutation of the regulator gene (*i*).

Table 11. The properties of the diploid obtained by transducing strain 147i (147: β i^+ p_C^+ Hg^R Em^S) with phage from the constitutive haploid segregant (strain 147 carrying the α (X35) Hg^S --- γ Em^R plasmid) of the diploid mutant X35. The properties of the parental strains and of the haploid segregants of the diploid are also shown.

Strain	Penicillinase activity (U/mg.)* Uninduced
Parental strains	
α-plasmid segregant of X35 (donor)	153
147i (recipient)	1
Diploid	6
Segregants	
X35 α-plasmid segregant	147
147i	1

* All penicillinase activities are the means of 3 values.

The other segregant from X35 had the resistance characteristics $\underline{Hg}^R$, $\underline{Em}^S$ and $\underline{Cd}^R$ of the β-plasmid of the $\underline{i}^+/\underline{i}^+$ diploid but had a penicillinase activity of less than 0·5 U/mg. uninduced or after induction. The regulator gene function of this segregant was tested by transducing into it the plasmid $\alpha\ \underline{i}^-\ \underline{p}_A^+\ \underline{Cd}^S$ --- γ $\underline{Em}^R$ from the stock magno-constitutive strain PC1 $\underline{Cd}^S\ \underline{Em}^R$. Six diploid clones doubly resistant for cadmium and erythromycin were compared for penicillinase production with the stock strain PC1 $\underline{Cd}^R\ \underline{Em}^R$ by staining streaks grown on the surface of agar plates with penicillin-iodine reagent. Cadmium and erythromycin were included in the medium to minimise the number of segregants present. No repression of constitutive penicillinase synthesis was observed. The plasmid carried by this segregant is concluded to have lost the activity of both $\underline{i}$ and $\underline{p}$ genes, possibly because of a deletion covering the penicillinase region.

The behaviour of the mutant diploid, X35, and its segregants, leads to the conclusion that the mutagenic treatment has affected both plasmid components of the diploid. One of the plasmids has been mutated from the $\underline{i}^+$ to the $\underline{i}^-$ state, and the level of penicillinase resulting from this mutation is expressed in the original diploid isolate because of the absence of an $\underline{i}^+$ gene on the other plasmid, and not because of an $\underline{O}^c$ mutation.

5. CONCLUSIONS

Regulator gene mutations were readily obtained after EMS and X-ray treatment of the $\underline{i}/\underline{i}$ diploid but no $\underline{O}^c$ mutants were

P_{45}^- is: i^+, low enz act'y

all "revertants" to high activity are thermolabile enzyme & presumed i^-

one much examined in detail was called "s/c s^-" & was $\underline{i^-}$ $\underline{p^-}$

Batch cultures of s/c s^- lysed ? difficult to get high cell density. "ala-lys" peptide in preps. < S

P_2 i

Cd Em.

isolated. Clones picked for having an increased penicillin activity were found to belong to the following categories: (a) Haploid strains; (b) $\underline{i}^+/\underline{i}^-$ diploid strains; (c) strains unstable for plasmid maintenance; (d) strains with mutations affecting both plasmids.

(a) Haploid strains

Many derepressed clones isolated no longer had both the resistance markers used to tag the two plasmids and were assumed to have lost one whole plasmid and now to be showing haploid expression of regulator constitutivity.

(b) $\underline{i}^+/\underline{i}^-$ diploid strains

Among the isolates shown to be still diploid, 10 from the EMS series and 1 from the X-ray treatment had an $\underline{i}^-$ mutation on one plasmid segregant and had in the diploid state the slightly raised basal enzyme levels of a repressed $\underline{i}^-$ constitutive.

(c) Strains unstable for plasmid maintenance

Five isolates from the EMS mutation appeared unstable as diploids, resulting in a raised basal level of enzyme due to the large numbers of constitutive segregants present. Diploids of this type have been described by Richmond (1967a), and arise from mutation of a host cell site controlling plasmid maintenance, alteration of which leads to rapid plasmid loss.

(d) Strains with mutations affecting both plasmids

Three EMS and two X-ray isolates were presumed to be still diploid on the basis of the presence of both the Hg and Em markers used to lable each of the two parental plasmids. One of these

plasmids was now constitutive and its penicillinase was fully expressed in the diploid isolate. When an EMS mutant, E14, and an X-ray mutant, X35, were studied in detail, the constitutive segregants from both were found to be repressible in heterogenotes with an $\underline{i}^+$ allele. However, in a small proportion of clones obtained with the E14 segregant in the cytoplasm of the mutant isolate, constitutivity was still expressed. In these clones recombination had occurred between the $\underline{\beta}$-plasmid from E14 and the $\underline{Em}$ region from the $\underline{\alpha}\ \underline{i}^+$ --- $\underline{\gamma}\ \underline{Em}^R$ plasmid to produce a new haploid clone with the exclusion of the $\underline{\alpha}\ \underline{i}^+\ \underline{p}_A^+$ loci. The other plasmid segregant from both these mutants, E14 and X35, was apparently altered or deleted over the whole penicillinase region. No enzyme activity was detected in either segregant, and there was no evidence of the A-type penicillinase expected of this plasmid from E14, neither alone nor in the original diploid isolate. These segregants did not show any repressor activity, either in the original mutants or in heterogenotes with test magno-constitutive regulator mutants. Thus the constitutive segregant from E14 and X35 had regulator gene mutations, $\underline{i}^-$, and the other segregants showed no evidence of $\underline{i}^-$ and $\underline{p}$-gene activity. This might be due to double mutation affecting both loci, but, as both mutagens can cause deletions, it is likely that this region has been lost from one of the plasmids.

No mutants of the operator region for penicillinase were isolated. It is possible that the mutation rate in this region is so low that insufficient numbers of colonies have been screened.

The method of detecting mutants affecting penicillinase is by scanning the total viable population, and not by selection of the mutants (see Section II.9.(d)) concerned as is possible with mutations of nutritional markers, and this means that a smaller population of bacteria is examined at any one time. Alternatively, if the rate of mutation to $\underline{O}^c$ is small compared to mutation rates for other sites on the plasmid, then this mutant may not be detected because of simultaneously occurring mutation such as loss of the structural gene activity or loss of the plasmid itself. From the experiments reported above, the mutation rate to $\underline{O}^c$ is less than 10^{-5} with X-rays and between 10^{-4} to 10^{-5} with EMS, whereas regulator gene mutation occurs at about 10^{-3} with EMS and at a detectable rate with X-rays. Mutations causing total or partial loss of a plasmid were also readily found within the scope of these experiments.

V. MICRO-CONSTITUTIVE MUTANTS

1. INTRODUCTION

Micro-penicillinase mutants are defined as mutants that have a basal level of activity less than that of the wild type strain, and such mutants have been found with activities ranging from 0·1 to 50% of the normal basal level. These mutants are either micro-inducible, with a normal induction ratio (about 30), or are micro-constitutive, with an induction ratio of less than 5 (Novick, 1963).

Richmond (1965c, 1966a & b) examined a group of micro-mutants which included both the micro-inducible and the micro-constitutive phenotypes. He showed that these arose from point mutations, which were not located in the penicillinase structural gene. Neither did this mutation lie in the regulator (i) gene since both types of mutant gave rise to the normal magno-inducible phenotype in diploids formed with a β i^- p_C^+ plasmid. Furthermore, the restriction in enzyme synthesis by the p-gene *cis* to the micro-mutation is not alleviated by the presence of the wild type allele *trans* in the same cell. Both the micro-inducible and the micro-constitutive loci appear to map between i_1^- and p_2^-. Richmond postulated a control region between i and p which might be of the type of the original Jacob & Monod (1961) operator, responsible both for repressor recognition and for initiation of operon expression. Novick (1963) also found a different type of micro-constitutive mutant (pen-mc-29) which reverted spontaneously to a high level constitutive form.

These findings suggested that further study of the genetic constitution of micro-constitutive mutants might help to determine the mode and site of action of the repressor, and therefore mutant P45 has been investigated. This mutant was originally isolated by Novick (1963) and called by him pen-mc 45. The mutant was isolated at the same time as pen-mc 29, and both were found by Novick to give rise to meso-constitutive revertants.

Mutant P45 was isolated after EMS treatment of the wild type strain 8325: $\underline{a}\ \underline{i}^{+}\ \underline{p}_{A}^{+}$. It makes 0·3 - 0·6 U/mg. penicillinase uninduced and 0·6 - 1·2 U/mg. induced, with an induction ratio of 2. Originally, the markers for $\underline{Cd}^{R}\ \underline{Hg}^{R}\ \underline{Em}^{S}$ were linked to the penicillinase genes, but for use in the experiments below the alleles $\underline{Cd}^{S}\ \underline{Em}^{R}$ were recombined onto the plasmid (for method, see Section II.11.(c).(ii)) to give a $\underline{Cd}^{S}\ \underline{Hg}^{R}\ \underline{Em}^{R}$ genotype.

2. PRELIMINARY DESCRIPTION OF MUTANT P45

The small amount of enzyme protein made by mutant P45 reacted with anti-A serum with some increase in enzymic activity, but it was not possible to use this reaction to determine whether the protein was a mutein or not, since the assay method was not sufficiently accurate to deal with the low levels of activity available for these experiments. It was therefore not clear whether the p-gene of P45 is mutated or not.

If strain P45 carried both regulator and structural gene mutations which caused the production of large amounts of enzyme

protein that is inactive or less active than the normal enzyme protein, then this should be detectable since the mutant protein, which was found above to be CRM^+, would interfere in the reaction of normal enzyme with its antiserum. No such interference was detected even when a tenfold larger volume of P45 supernatant was present (M.H. Richmond and P. Thompson, unpublished experiments). Hence P45 does not make constitutive quantities of an enzyme protein of very low activity.

The properties of the diploid formed by transducing the P45 plasmid into a $\underline{\beta}\ \underline{i}^-\ \underline{p}_C^+$ strain, 147C223, demonstrated that P45 is $\underline{i}^+$. The diploid made 4 U/mg. basal enzyme and 160 U/mg. after induction, giving an induction ratio of 40. This is consistent with full repression of the $\underline{p}_C^-$ gene and restoration of full inducibility to the diploid.

The proportion of exoenzyme released from the induced diploid was within the range found in individual experiments for 147C223 alone, so that it would seem that if the mutant contains a normal $\underline{p}_A$-gene, it is not fully expressed.

To test the possibility that the low enzyme activity of strain P45 might be due to production of a penicillinase-destroying or modifying factor, equal inocula of P45 and PC1 were incubated together for 4 hr. at 37°. The mixture was assayed for penicillinase and for the proportion of PC1 colony forming units. The total observed penicillinase activity was close to that expected from the strain composition of the mixture at the end of the experiment, as was the amount released as extracellular enzyme.

This latter could not be distinguished from normal A-type enzyme on the basis of heat inactivation at 40° (in contrast to the revertants and recombinants of P45 discussed below). This evidence makes it unlikely that the low enzyme content of the micro-constitutive mutant is due to the production of a factor that can destroy or inactivate penicillinase formed by unmutated strains.

The phenotype of P45 was unchanged in over 100 transductant colonies obtained by transduction of the P45 plasmid into the unmutated, plasmid-negative, host strain, 8325 N.

From the preliminary experiments it was concluded that the changes giving rise to the P45 phenotype were due to events within the penicillinase region. Further information could be obtained by the following approaches: a) reversion, b) recombination, and c) complementation.

3. REVERSION TO INCREASED PENICILLINASE ACTIVITY

Further mutation might give information about the original mutation. For example, a point mutation could revert to wild phenotype, whereas a deletion or multisite mutation would not do so. Enzymic activity might be restored to a structural gene mutant, though not necessarily by restoring the original base sequence of the gene determining it so that an active but structurally altered protein is produced.

P45 was treated with mutagens, and plated on agar containing

erythromycin to eliminate plasmid negative clones and mutants of the Em-gene. Clones with penicillinase activities above the micro level were regarded as revertants. Revertants were isolated from three separate experiments directed at determining the effects of two different mutagens, NG and EMS, one of which (EMS) might be expected to reverse an EMS-induced forward mutation (Krieg, 1963), and at isolating mutants under conditions allowing detection of changes either in the basal or in the induced level of penicillinase expression.

(a) The Revertants

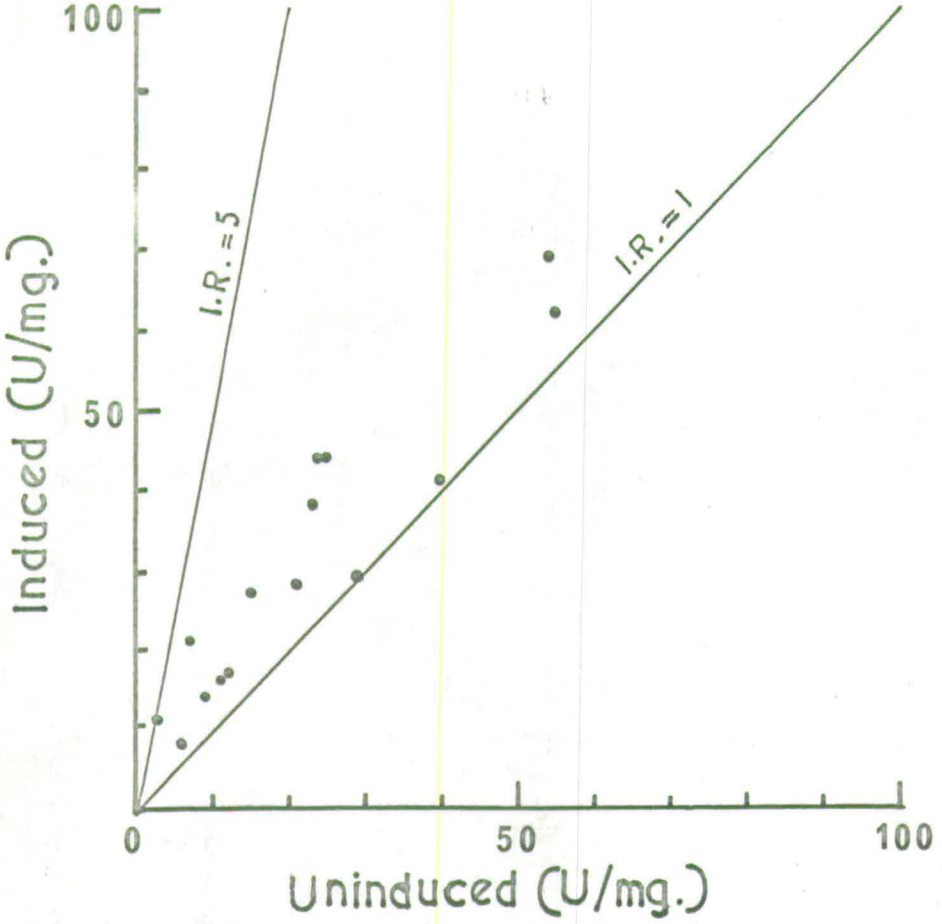
(i) NG-induced mutation.

Mutants with increased basal activity were obtained after NG treatment. These arose at a frequency of about $5/10^3$ survivors. In all, 15 revertants (in the series of isolates N1-N18) were isolated and their phenotypes are represented by the scatter diagram Fig. 5a. The uninduced penicillinase activities of these mutants were found to range from the wild-type level to about 60 U/mg., the highest being about 20% of the constitutive level. All revertants were constitutive, with an induction ratio in all cases of less than 5.

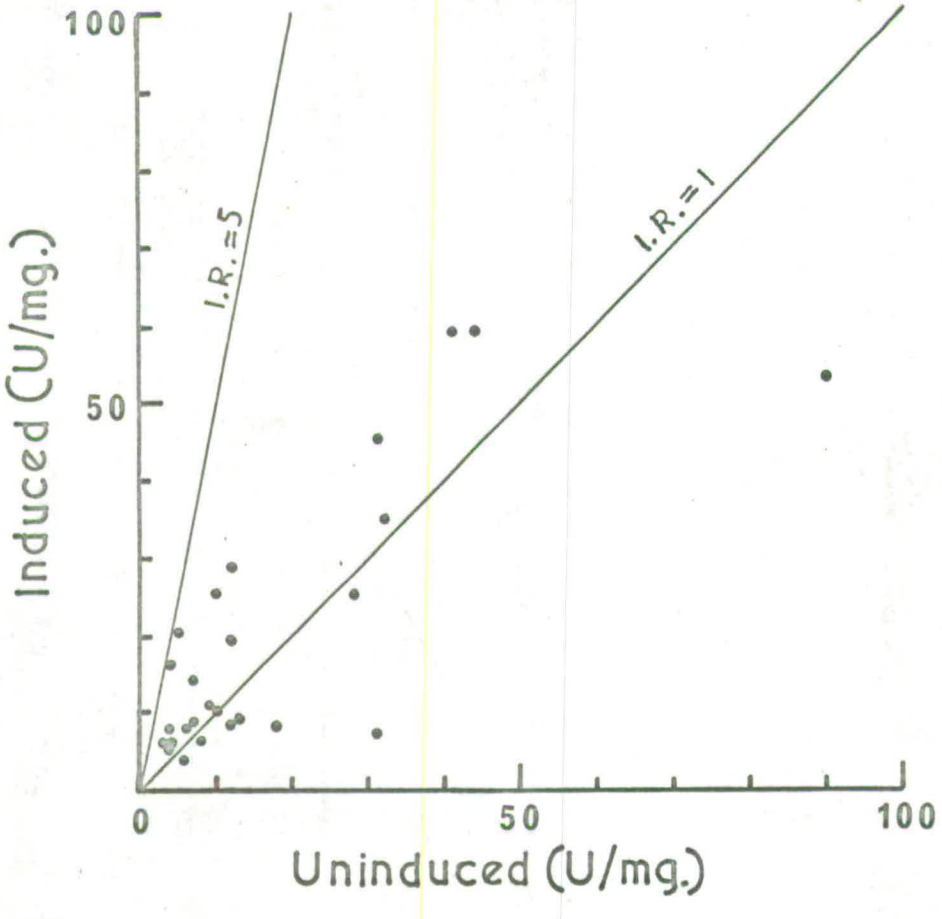
(ii) Mutants isolated under induction conditions after NG treatment.

Fully inducible revertants were sought by treating P45 with NG and screening for increased enzyme levels on agar containing inducer (cephalosporin C). Fifty-four possible isolates were made (N23-N77) and Fig. 5b shows the phenotypes of the 26 revertants

(a)


100
50
0
50
100
Induced (U/mg.)
Uninduced (U/mg.)
I.R.=5
I.R.=1

(b)


100
50
0
50
100
Induced (U/mg.)
Uninduced (U/mg.)
I.R.=5
I.R.=1

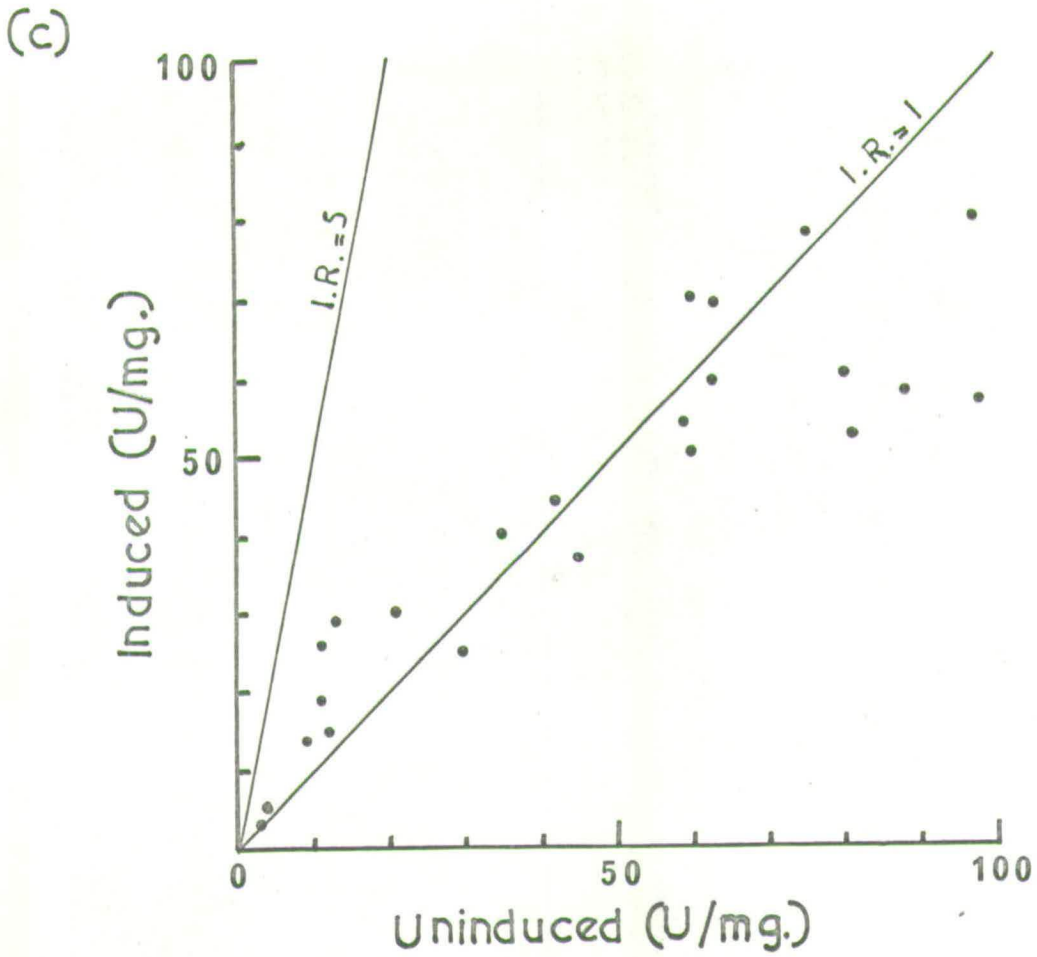


Fig. 5. Scatter diagrams showing the uninduced and induced levels of penicillinase activity in revertants to increased enzyme production of the micro-constitutive mutant P45. The results of three independent experiments are shown. In (a) and (b) revertants were produced with NG, and in (c) with EMS. Each mutant is represented as a point located on the abscissa by the uninduced enzyme level and on the ordinate by induced enzyme level. The diagonal lines divide the diagram into areas of I.R. of 0 - 1, 1 - 5 and 5 - ∞.

obtained. These phenotypes were similar to the first series, the difference in the size of phenotype clusters between the two series of isolates being probably due to selection bias. No revertant was significantly inducible, all induction ratios being less than 5. Maximal enzyme level attained was again about 20% of the wild type maximum.

(iii) EMS-induced mutation.

A further twenty-four revertants (in the series of isolates EMS 1 - 60) to increased penicillinase activity were obtained by EMS mutation, and selection in the presence of inducer. The properties of these isolates (Fig. 5c) show a similar range of basal enzyme activities from about normal to about 80 U/mg. similar to the NG series. The greatest enzyme level obtained was about 25% of normal, and again there was no significant induction of any revertant. Reversion frequencies of about $1\text{-}5/10^3$ were found with EMS.

These experiments showed that no magno-constitutive and no inducible revertants are obtained by further mutation of strain P45.

(b) The structural gene of revertants of strain P45

The meso-constitutive phenotype of the revertants of strain P45 could be due to changes in the regulator region or in the structural gene. To elucidate this situation, one revertant, N3, was chosen for examination in greater detail. The mutational events responsible for the N3 phenotype are plasmid linked and not a characteristic of the host cell as shown by transduction of N3

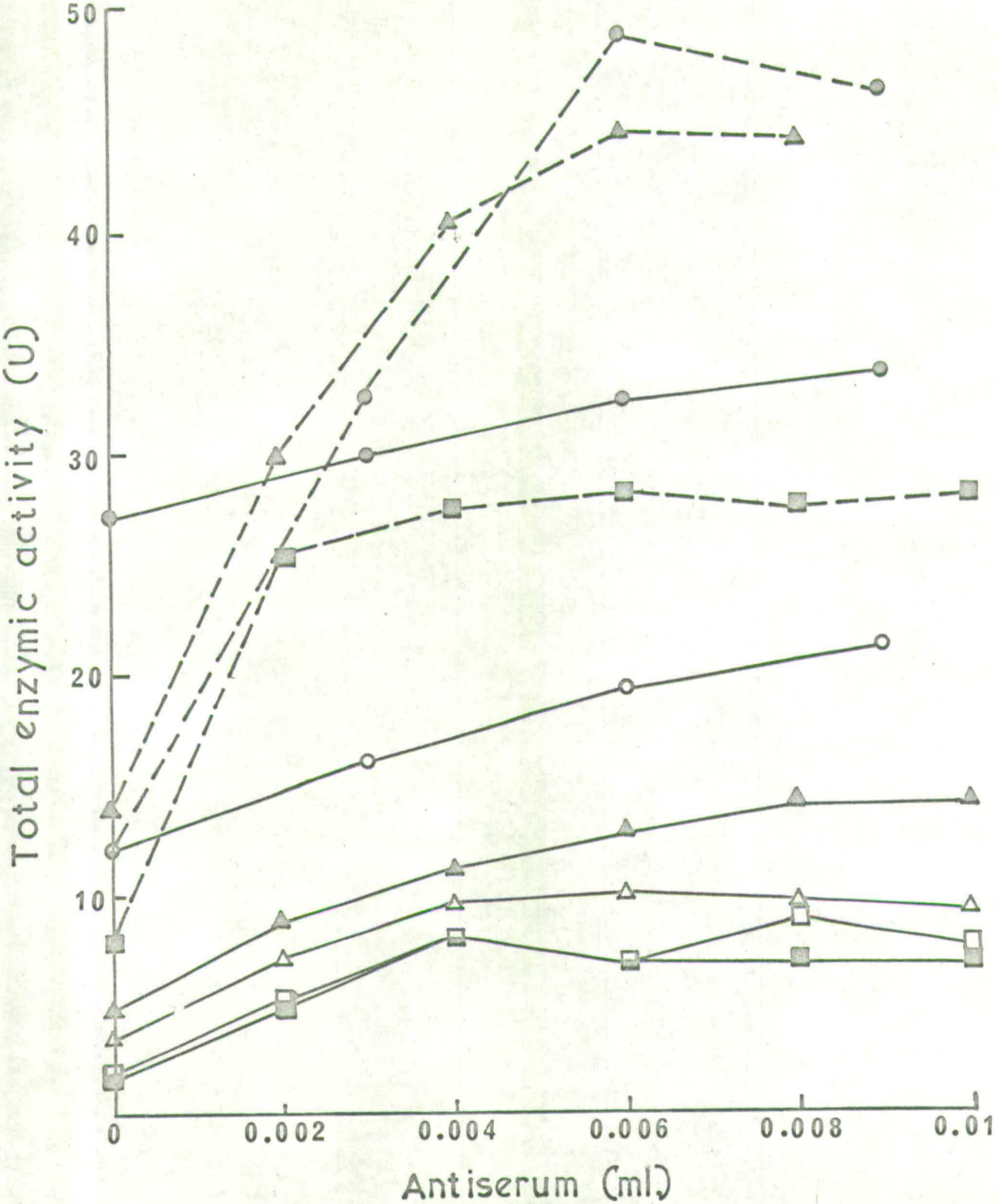
into 147N (Table 13, below). The reaction of the exoenzyme of N3 with antiserum indicated that the enzyme was mutein and if the protein-antibody interaction is unaffected, this had a relative activity of about 20% of normal (Fig. 6).

Preliminary experiments also showed that at temperatures up to 50°, N3 exoenzyme was inactivated faster than PC1 exoenzyme. It was possible to differentiate clearly between N3 and PC1 supernatant enzyme at 40°, (Fig. 7), and a representative group of the other revertants was screened for enzyme instability at this temperature. Tubes containing equal volumes of the supernatants of the test cultures and of PC1, suitably diluted, were simultaneously placed in a water bath at 40° and simultaneously removed after 12-13 min. to a bed of crushed ice. In this way, test and control material were handled as similarly as possible. Enzyme activities before and after heating were determined. The results for NG and EMS mutants are given in Tables 12a and b and show that all the revertants tested have a relatively heat labile enzyme. The tests were carried out in CHY medium which was buffered to pH 7·4 and was found to be still at this pH value after staphylococcal growth.

(c) The regulator gene of revertants of P45

The meso-constitutive revertants of P45, such as N3, may have residual sensitivity to the repressor as well as residual repressor activity. The first possibility was tested by complementation with an i^+ allele in the *trans* position, and the second with an i^- allele *trans*.

Fig. 6. The antigenic reaction of the extracellular penicillinase from the revertant N3 and the recombinant rec5 of the micro-constitutive strain P45 compared with the reaction of unmutated A-type enzyme from strain PC1. The antiserum was prepared against purified A-type penicillinase (Richmond, 1963). The results of 3 experiments are shown in the following manner:
O—O, △—△, □—□, N3; ●—●, ▲—▲, ■—■, rec5; ●---●, ▲---▲, ■---■, PC1.
The experimental details are given in the text.


50
40
30
20
10
Total enzymic activity (U)
0
0.002
0.004
0.006
0.008
0.01
Antiserum (ml)

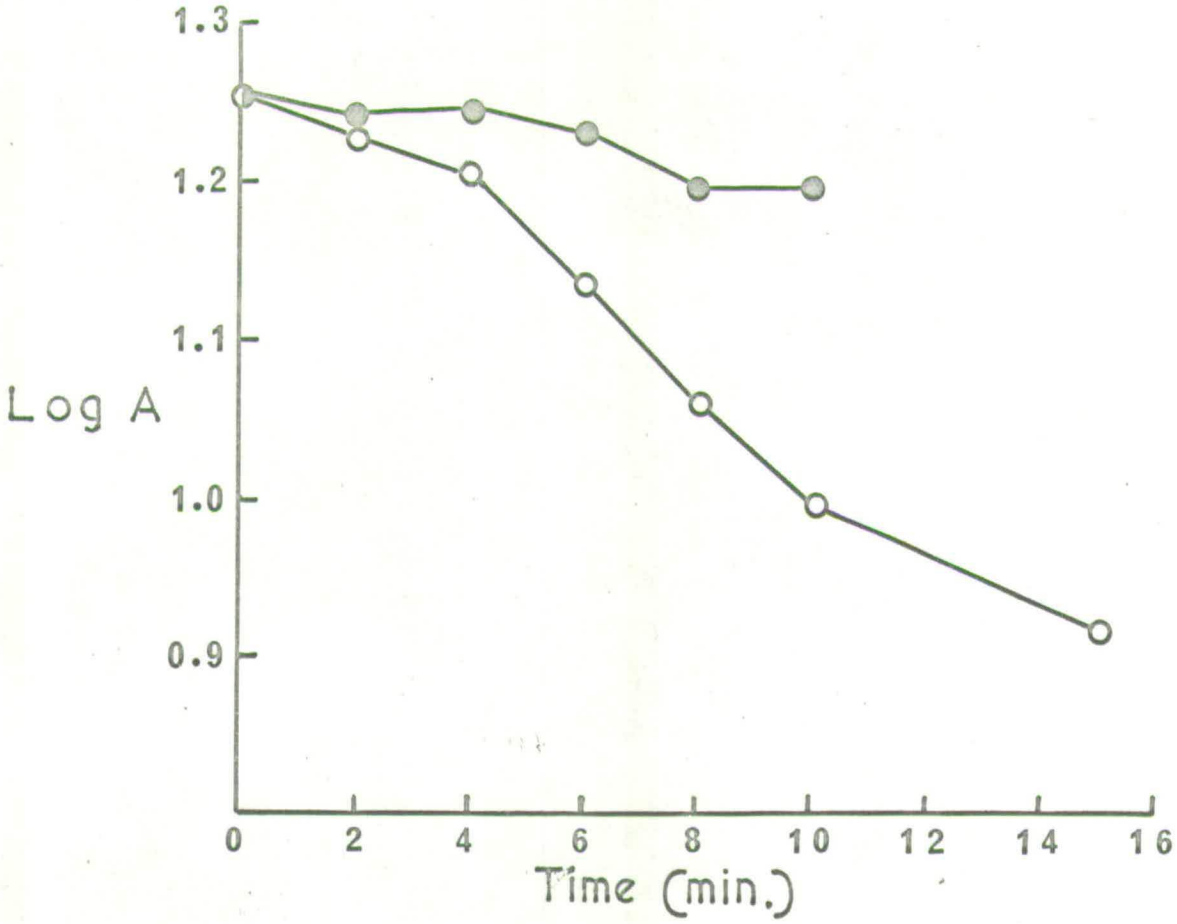


Fig. 7. The rate of heat inactivation of the extracellular penicillinase from the revertant N3(○) of the micro-constitutive mutant P45 and of the unmutated A-type exoenzyme from the strain PC1(●) at 40°. A is the activity of the exoenzyme in U/ml.

Table 12. The comparative temperature sensitivity of the extracellular penicillinase of different revertants to increased enzyme production of the micro-constitutive strain P45.

The results of two experiments are shown, (a) and (b). In (a) the revertants were produced by NG mutation, and in (b) by EMS. In each experiment the results for unmutated A-type penicillinase from strain PC1 are also shown.

(a) Heating: 12 min. at 40°			(b) Heating: 13 min. at 40°		
Strain	Total uninduced penicillinase activity (U/mg.)	Coefficient* of inactivation of the exoenzyme	Strain	Total uninduced penicillinase activity (U/mg.)	Coefficient* of inactivation of the exoenzyme
Revertant enzyme					
N1	54	-99	EMS 1	97	-49
N3	25	-82	EMS 10	88	-43
N5	24	-83	EMS 45	77	-69
N9	15	-29	EMS 2	75	-91
N2	7	-33	EMS 55	63	-51
N6	6	-37	EMS 13	60	-59
N7	3	-30	EMS 31	59	-55
N46	10	-34	EMS 60	45	-27
N32	5	-34	EMS 19	30	-84
N54	4	-32	EMS 44	17	-61
N68	4	-25	EMS 21	12	-48
Unmutated enzyme (A-type)					
PC1 (i)≠		-15	PC1 (i)≠		-8
(ii)		-11	(ii)		-5
(iii)		-15	(iii)		-9

*Coefficient of inactivation = $1000 \times \frac{\text{log final activity - log initial activity}}{\text{time of heating (min.)}}$

≠ PC1 (i)-(iii) are dilutions in CHY medium of the unmutated exoenzyme of strain PC1 to initial activities similar to those of the revertant exoenzymes.

The sensitivity of the rate of enzyme synthesis of the revertant N3 to the normal repressor molecule is shown by the properties of the diploid formed by transduction of N3 into 147i ($\underline{\beta}\ \underline{i}^{+}\ \underline{p}_{C}^{+}$), as given in Table 13.

The induction of N3 penicillinase in the presence of normal $\underline{i}^{+}$ allele was investigated. It was not possible to use the anti-serum reaction method to measure the change in N3 enzyme content in the supernatant of diploid cultures because of the altered immunological properties of the N3 penicillinase (Fig. 6), but an estimate of the extent of expression of the penicillinase gene of the $\underline{\alpha}$-plasmid in an induced diploid (147: $\underline{\alpha}$ (N3)/$\underline{\beta}\ \underline{i}^{+}\ \underline{p}_{C}^{+}$) was made from the ratio of exoenzyme to cell-bound enzyme. The details of this test and an interpretation of the results are discussed in the section on complementation tests for induction of P45 enzyme (Section V.5). The results are shown in Table 14, control measurements being made at the same time on the parent strains and segregants, and are consistent with full induction of N3 enzyme.

Attempts were made to test N3 for residual repressor activity by transduction of its plasmid into 147C223 ($\underline{\beta}\ \underline{i}^{-}\ \underline{p}_{C}^{+}$) to form a plasmid diploid, but the results were erratic and difficult to interpret. In the first experiment two diploid clones, possibly sibs, were obtained. Cultures from single colony isolates of one of these made about 130 U/mg. after induction and had basal levels of about 15 U/mg., or 8 U/mg. when the observed values were corrected for the haploid magno-constitutive ($\underline{\beta}$C223) segregants present. Haploid segregants from which the $\underline{\alpha}$-plasmid has been

Table 13. The properties of diploid strains obtained by transducing strain 147i (147: β i^+ p_C^+ Cd^R Em^S) with phage from the revertant N3 (8325: α (N3) Cd^S --- γ Em^R) of strain P45, and of the transductant strain obtained by transducing the plasmid-negative host strain 147 N with phage from the revertant N3. The two diploid strains were constructed in independent experiments. The properties of the parental strains and the haploid segregants of the diploids are also shown.

Strain	Penicillinase activity (U/mg.)*		Induction ratio
	Uninduced	Induced	
Parental strains			
N3 (donor)	26	35	1·4
147i (recipient)	1	122	122
Diploid (1)	2	72	36
Segregants from diploid (1)			
147:N3/	33	27	0·8
147i	2	105	53
Diploid (2)	1	83	83
Segregants from diploid (2)			
147:N3/	32	39	1·2
147i	1	94	94
Transductant (N3) in host strain 147	35	29	0·8

* Mean of 3 values in each case.

/ 147:N3 has the characteristics of the α-plasmid from N3 in the host strain 147.

Table 14. The properties of induced cultures of diploids constructed by transducing the wild type inducible strain 147i (147: $\underline{\beta}\ \underline{i}^{+}\ \underline{p}_{C}^{+}\ \underline{Cd}^{R}\ \underline{Em}^{S}$) with phage from the NG-induced revertant N3 (8325: $\underline{\alpha}$ (N3) $\underline{Cd}^{S}$ --- $\underline{\gamma}\ \underline{Em}^{R}$) of strain P45 (Table 13).

The properties of the parental strains, two diploids constructed independently, and haploid segregants from the diploids are shown.

The total and exoenzyme activities of the diploid strains are shown for whole cultures assayed by the Perret method, and for the diploid population in these cultures, calculated from the assayed values by correcting for the activities of the haploid segregants present in each culture.* The values of the haploid activities used in the calculation were those obtained for the relevant segregants; for the results in parenthesis, the values of the parental strains were used.

Strain	Penicillinase activities of induced cultures (assayed values)/			Penicillinase activities corrected for the number of segregants in the assayed culture*/		
	Total enzyme (U/mg.)	Exoenzyme (U/mg.)	Exoenzyme (% of total)	Total enzyme (U/mg.)	Exoenzyme (U/mg.)	Exoenzyme (% of total)
Parental strains						
N3 (donor)	28	17·2	61			
147i (recipient)	194	6·2	3			
Diploid (1)	90	8·7	10	90(76)	9·7(8·7)	10(11)
Segregants from diploid (1)						
147:N3 #	41	14·0	35			
147i	101	4·6	4			
Diploid (2)#	83	10·1	12	84(71)	10·6(10·5)	13(15)
Segregants from diploid (2)						
147:N3 ##	34	14·4	43			
147i #	93	5·9	6			

* The composition of the assayed cultures from diploid (1) was 83-94% diploid cells, 0-3% α-segregants, 3-17% β-segregants; and from diploid (2) the proportions of each type were respectively, 90-91%, 0·1% and 7-10%.

/ Unless otherwise stated the results are the mean of 3 values in each case.

147:N3 has the characteristics of the α-plasmid from strain N3 in the host strain 147.

Mean of 2 values in each case.

lost occur at about 10 times the frequency usual for the corresponding diploid carrying the wild type α-plasmid from P_o. These assays were carried out after the diploids had been stored at 4° for 3 weeks, followed by further subculturing and single clone isolation. Segregants, obtained 2 - 3 days after construction of the diploid, had enzyme levels characteristic of the parents, N3 and 147C223, i.e., 30 - 40 U/mg. and 150 - 200 U/mg. respectively. Further segregants of the diploid, isolated on the same day as it was assayed, also appeared to have enzyme activities like N3 and 147C223 by the penicillin-iodine test on colonies grown on CY agar. These results suggested complementation between two i^- alleles to form a functional repressor.

In subsequent transductions, most diploid clones were very unstable and segregated to the constituent haploid forms so rapidly that determination of the penicillinase level of the diploid was not possible. For example, by the time a culture had grown enough to assay, it was composed of 75% or more of haploid segregants. Occasionally, a more stable diploid clone could be isolated, and cultures of this clone were still composed of over 90% diploid cells when assayed. Like the diploids from the first transduction experiment, these isolates were inducible to 140 U/mg. and had a basal level of about 8 U/mg. at assay. Two segregants were obtained from each of these diploids, but the pattern of segregation differed from that discovered previously. One segregant, indeed, resembled the control strain 147C223, but the other, the probable α-segregant, isolated from the diploids

within 3 days of their assay, made less than 10 U/mg. These diploids had been prepared over a period of about two months, stored in the cold-room, and at the end of this time segregants were isolated and assayed with the diploid clone. The conclusion reached from the results of the assays is that the presumptive N3 segregant no longer has an enzyme level as high as the original N3 parent, and must have undergone further genetic modification. Because of this modification, repression of the enzyme level of trans allele in the diploid is now also possible.

Other NG revertants of P45 were transduced into 147C223. The reason for this was that several revertants isolated made low basal levels of enzyme and so may still have been making an effective repressor. Four such strains were tested by transduction into 147C223 and assay of the basal level of diploids formed. These diploids were all unstable. The values observed and corrected for the segregant population present are shown in Table 15. The results show two levels of repression, to about 150 U/mg. and to about 50 U/mg.; no fully repressed diploids were found.

The 147C223 stock culture used in these tests formed normal, repressed, stable diploids with the wild-type α-plasmid from P_o, and segregant colonies are present at less than 1% in most broth cultures.

Table 15. The properties of uninduced cultures of the diploids constructed by transducing the magno-constitutive strain 147C223 (147: $\underline{\beta}$ $\underline{i}^-$ $\underline{p}_C^+$ $\underline{Cd}^R$ $\underline{Em}^S$) with phage from each of the NG revertants, N6, N46, N54, N64 (8325: $\underline{\alpha}$ (N...) $\underline{Cd}^S$ --- $\underline{\gamma}$ $\underline{Em}^R$), of the micro-constitutive strain P45.

The penicillinase activities shown are for the whole culture assayed by the Perret method, and for the diploid cells in the culture, obtained by correcting the assayed value for the activities of the haploid segregants present in the culture. The properties are also shown of the parental strains, and of some haploid segregants from the diploids. The remaining segregants, all containing the $\underline{\beta}$-plasmid, were found to resemble 147C223 by comparison of clones grown on solid medium. The proportion of segregant cells in each culture was found by replica plating colonies, from samples of each culture, onto media singly selective for the resistance markers, $\underline{Cd}^R$ and $\underline{Em}^R$, which are carried separately by each plasmid.

Strain	Uninduced penicillinase activity (U/mg.)		Composition of assayed culture			
	Whole culture	Diploid cells	Diploid	α-segregant	β-segregant	Plasmid negative
Parental strains						
N6)	6					
N46) (donor)	10					
N54)	4					
N68)	4					
147C223 (recipient)	258					
Diploids*						
with N6	135	143	88-92%	5-10%	2-4%	<1%
with N46	134	163	79-90%	6-18%	3-4%	-
with N54	163	182	75-91%	3-19%	6-7%	-
with N68	63	60	74-75%	2-4%	3-4%	-
Segregants ≠						
147:N6	14					
147:N46	24					
147:N54	18					
147:N68	8					
147C223 (from the diploid with N6)	275					

* Mean of 2 values in each case.

≠ 147:N6, 147:N46, 147:N54 and 147:N68 have the characteristics of the α-plasmid from strains N6, N46, N54 and N68, respectively, in the host strain 147.

4. RECOMBINATION EXPERIMENTS WITH P45

Information on the genotype of P45 might be obtained by recombination of a mutated region into a plasmid of otherwise known genotype. If two or more mutated sites are involved, these might be separated and studied independently, and also, the state of, for example, the structural gene, can be investigated without the same risk of secondary modifications inherent in reversion experiments.

(a) Recombination with P_o, ($\underline{a}$ $\underline{i}^+$ $\underline{p}_A^+$)

The constitutivity of P45 may involve an $\underline{i}^-$ or an $\underline{O}$-region mutation which might confer high penicillinase activity when recombined into an otherwise wild-type plasmid. To test this possibility the plasmid from strain P45 $\underline{Cd}^S$ $\underline{Em}^R$ was transduced into P_o $\underline{Cd}^R$ $\underline{Em}^S$ and transductants selected for the marker $\underline{Em}^R$. Altogether 9,300 transductant clones were examined and none were high penicillinase producers. Therefore, P45 does not appear to carry an $\underline{i}^-$ or an $\underline{O}$-region mutation.

In this experiment transductants were selected on erythromycin agar after a period of incubation in CY broth. Although none were clearly constitutive, some colonies were examined further and many were of mixed phenotype, including that of the parental P_o $\underline{Em}^S$, even when plated after 24 hr. further incubation. In such colonies a magno-constitutive phenotype would have been detectable even if present as only 5% of the clone. A similar persistence of heterophenotype is being investigated by Dr. Richmond, and may be due to late phage infection during the

incubation period, or to partial protection in the growing clone of $\underline{Em}^S$ cells against erythromycin inhibition. For instance, when the plasmid negative, $\underline{Em}^S$ strain, P16, is densely plated on erythromycin-agar, growth similar to that of an $\underline{Em}^R$ strain occurs.

(b) Recombination with P2C ($\underline{\alpha}\ \underline{i}^-\ \underline{p}_2^-$)

Further evidence on the state of the structural gene for penicillinase in P45 was sought by linking it to a derepressed regulator gene. This came from the cross with strain P2C. The $\underline{\alpha}$-plasmid of this strain has independent mutations of the penicillinase structural gene, $\underline{p}_2^-$, and of the regulator gene, $\underline{i}_1^-$ (Richmond, 1966a); $\underline{p}_2^-$ is due to a single amino acid change at residue 40 (out of a total of 258) in the penicillinase protein (R.P. Ambler, personal communication), and has an enzymic activity of 1/20 of normal A-type penicillinase. The strain P2C is constitutive, making about 20 U/mg. uninduced and induced. Were P45 to be $\underline{p}^+$, then magno-constitutive recombinants would be expected, while if P45 were $\underline{pr}^-$ (promoter) (Jacob et al., 1964), then recombination to wild type, $\underline{pr}^+\ \underline{p}^+$, would be less likely because $\underline{p}_2^-$ is near the amino end of the protein, and hence probably close to the site of $\underline{pr}$. A recombinant of this sort would be recognized because the magno-constitutive level of gene expression could now be attained.

The plasmid from P45 $\underline{Cd}^S\ \underline{Em}^R$ was transduced into strain P2C $\underline{Cd}^R\ \underline{Em}^S$, and $\underline{Em}^R$ transductants were selected after overnight incubation to allow segregation, and screened for penicillinase production. No magno-constitutive recombinants were found in a

total of 10,500 $\underline{Em}^R$ transductants. However, meso-constitutive recombinants were obtained, 17 (rec 1 - 17) being isolated from one experiment of 4,200 $\underline{Em}^R$ transductants. Using a fresh phage preparation an independent recombinant (rec a) was found among 750 transductants. These recombinants had an enzyme level slightly but significantly greater than P2C (Table 16). Some recombinants were first isolated from clones of mixed phenotypes, so it is possible that some were not noticed and the actual recombination rate was higher than these results indicate.

As a control for the transduction process in the recombination experiments, the plasmid from P45 $\underline{Cd}^S$ $\underline{Em}^R$ was transduced into the negative recipient, P16. No magno- nor meso-constitutive clones were found amongst 10,800 $\underline{Em}^R$ transductants, all of which had an enzyme level similar to the original P45 donor strain.

(c) Properties of the P45 x P2C meso-constitutive recombinants

All the meso-constitutive recombinants were $\underline{Cd}^S$ like the P45 parent, and all appeared to make similar amounts of enzyme. The assayed values of three from the first series of recombinants, rec 5, rec 9, rec 12, and of the independently obtained recombinant, rec a, are shown in Table 16. The recombinants make 50 - 60 U/mg. basal penicillinase and have an I.R. of about 1.

The level of penicillinase production is unchaged by transduction into a plasmid-negative host, P16, showing that the genes determining this level are plasmid linked (Table 16).

Table 16. The properties of the meso-constitutive recombinants from the transductional cross of P45 x P2C.

The recombinants were obtained by transducing the strain P2C (8325: $\underline{a}$ $\underline{i}^-$ $\underline{p}^-_{A2}$ $\underline{Cd}^R$ $\underline{Em}^S$) with phage from the micro-constitutive strain P45 (8325: $\underline{a}$ (P45) $\underline{Cd}^S$ --- $\underline{\gamma}$ $\underline{Em}^R$). Recombinant strains rec 5, rec 9 and rec 12 were isolated in one experiment, and rec a in an independent experiment. The properties are also shown of the parent strains P45 and P2C and of transductant clones obtained when the plasmid negative strain 8325 N is transduced with phage from the recombinant rec 5.

Strain	Penicillinase activity (U/mg.)* Uninduced	Induced	Induction ratio
Parental strains			
P45 (donor)	0·5	1·1	2·2
P2C (recipient)	18	16	0·9
Recombinants			
rec 5	64	53	0·8
rec 9	63	46	0·7
rec 12	67	48	0·7
rec a	53	56	1·1
Transductants			
(rec 5) in strain 8325 N (1)	57	53	0·9
(rec 5) in strain 8325 N (2)	55	49	0·9

* Mean of 2 values in each case.

(i) The structural gene of the recombinants

Exoenzyme from the recombinants is more temperature sensitive than exoenzyme from PC1 and P2C. This is similar to the result for P45 revertants (above, Section V.3.(b)), and the experiments were carried out in a similar way. The increased temperature-sensitivity was similar for both independently obtained recombinants, rec 5 and rec a, and for rec 5 after transduction into the different plasmid-negative host, P16 (Table 17).

The antigenic response of rec 5 enzyme had the characteristics of a mutein with an enzymic activity of about 20% of the wild type enzyme (Fig. 6), provided that the protein-antibody interaction is unchanged.

(ii) Purification of rec 5 enzyme

The exoenzyme from rec 5 was readily absorbed to cellulose-phosphate, not apparently removed during the washing process, but only 10 - 15% of the activity was recovered after ammonium sulphate elution, and the recovery after gel-filtration through Sephadex G-100 was only 1 - 5%. The normal staphylococcal A-enzyme and mutant enzyme P2 are both typically obtained in about 75% and 90% yield at the two steps. Actual recoveries in two large scale (10 l.) preparations are shown in Table 18. Small scale experiments were undertaken to try to find better conditions for elution from the cellulose phosphate, but without success.

The fractions eluted off the Sephadex G-100 and containing the small amount of enzymic activity were pooled, and the protein present examined by high voltage paper electrophoresis after

Table 17. The comparative temperature sensitivity of the extracellular penicillinase of several meso-constitutive recombinants from the transductional cross P45 x P2C (Table 16).

The results shown are of two experiments, (a) and (b), with enzyme from the parental strain P2C, some recombinant strains, and the unmutated A-type enzyme from strain PC1. The recombinant rec a was isolated independently of the recombinants rec 5, rec 9 and rec 12. In (b) the results are also shown of two transductant clones obtained by transducing strain 8325 N with phage from strain rec 5. The second parental strain P45 has insufficient penicillinase activity for includion in these experiments.

Strain	Heating: (a) 12 min. at 40° Coefficient* of inactivation of exoenzyme	Heating: (b) 10 min. at 40° Coefficient* of inactivation of exoenzyme
Parental strain		
P2C	-4 -7	+1
Recombinants		
rec 5	-21	-39
rec 9	-24	
rec 12	-22	
rec a		-39
Transductants		
(rec 5) in strain 8325 N (1)		-35
(rec 5) in strain 8325 N (2)		-32
Unmutated enzyme (A-type)		
PC1 (i)≠	-13	+3
(ii)	-11	
(iii)	-10	

* Coefficient of inactivation =

$$1000 \times \frac{\text{log final activity - log initial activity}}{\text{Time of heating (min.)}}$$

≠ The exoenzyme from strain PC1 is diluted in CHY medium to initial activities, (i)-(iii) in experiment (a) and (i) in experiment (b), similar to those of the other exoenzymes included in the experiment.

Table 18. Summary of the purification of the extracellular penicillinase of recombinant strain rec 5.

Experimental details are given in the text.

Stage of purification	Preparation 1		Preparation 2	
	Total enzyme activity (10^{-3}U)	Recovery (%) per stage	Total enzyme activity (10^{-3}U)	Recovery (%) per stage
Total exoenzyme (10 l.)	1,150		624	
Not absorbed to cellulose phosphate	100		52	
(Absorbed to cellulose phosphate, by difference)	(1,050)		(572)	
Eluted from cellulose phosphate	144	12·5%	67	10·7%
Eluted from Sephadex G-100	1	0·7%	3	4·5%

tryptic digestion. None of the characteristic acidic peptides of the staphylococcal penicillinase (which include both the C-terminus and N-terminus of the molecule) were detected, and the principal acidic peptide was probably the same as the principal acidic tryptic peptide from the X-protein, a known neighbour of penicillinase in this elution pattern.

(iii) The regulator gene of rec 5.

The postulate that rec 5 had a regulator gene $\underline{i}^-$ allele was confirmed by the nature of the diploid formed when it was transduced into 147i. Repressed enzyme levels of 2 and 4 U/mg. were produced by two such diploids.

These diploids induced to 140 - 240 U/mg., the induction of rec 5 enzyme being estimated from the proportion of exoenzyme (see Section V.5). The measured value was 9%, but when allowance is made for segregants present becomes 10 (13)% from diploid bacteria (59% of culture, the remainder being $\underline{\beta}$-plasmid segregants). Haploid rec 5 enzyme is 50% extracellular, 147i (segregant) is 7%, and 147i (stock) is 5% in the same experiment. From the proportion of penicillinase activity released as exoenzyme from the induced diploid, it was concluded that the $\underline{p_A}^-$ gene of rec 5 is fully induced.

5. INDUCTION OF P45 IN COMPLEMENTING DIPLOIDS

Inducible diploids were formed when a P45 plasmid was transduced into strain 147 containing the $\underline{\beta}\ \underline{i}^+\ \underline{p_C}^+$ or $\underline{\beta}\ \underline{i}^-\ \underline{p_C}^+$ plasmid. P45 enzyme might be induced at the same time, but would

not be readily detectable if it is a mutein. As A- and C-penicillinases retain, in diploids, their haploid characteristics for release into the medium as exoenzyme, the proportion of this enzyme from the induced diploid will reflect the induction of mutant A-enzyme. A normal $\underline{\alpha}\ p_A^+/\underline{\beta}\ p_C^+$ diploid inducing to 200 U (100 A + 100 C) will release 55 U (50 A + 5 C) = 28%. If the mutant A-enzyme has 1/5 normal activity per mole, then the induced diploid, making equal amounts of A & C protein, has an activity of 120 U (20 A + 100 C) of which 15 U (10 A + 5 C) = 13% is extracellular.

The proportion of enzyme released into the supernatant by the diploids with P45 was measured and compared with that of induced $\underline{\beta}$-plasmid haploids assayed in the same batch of medium at the same time. P45 when alone makes 0·7 - 1·2 U/mg. induced of which 30 - 50% is extracellular.

It is seen from Table 19 a and b that the stock strains of 147i and 147C223 produce 4 - 5% exoenzyme, $\underline{\beta}$-plasmid segregants produce 4 - 5%, and the diploids asaay at 7 - 8% which, after correction for segregants present, becomes 7 - 9% (8 - 11%). There were considerable differences in the results obtained from individual experiments, probably due to variations in the media.

Table 19. The properties of induced cultures of the diploids constructed with phage from the micro-constitutive strain P45 (8325: $\underline{\alpha}$ (P45) $\underline{Cd}^S$ --- $\underline{\gamma}$ $\underline{Em}^R$).

The results of two experiments are shown, (a) & (b). In (a) two diploids were obtained independently by transducing the wild type inducible strain 147i (147: $\underline{\beta}$ $\underline{i}^+$ $\underline{p}_C^+$ $\underline{Cd}^R$ $\underline{Em}^S$) with phage from strain P45. In (b) the diploid was obtained by transducing the magno-constitutive strain 147C223 (147: $\underline{\beta}$ $\underline{i}^-$ $\underline{p}_C^+$ $\underline{Cd}^R$ $\underline{Em}^S$) with phage from strain P45.

The properties are also shown of the parental strains and the haploid, $\underline{\beta}$-plasmid segregant of the diploids. The penicillinase activities of the parental strain P45 and of the $\underline{\alpha}$-plasmid segregants of the diploids were too small (about 1 U/mg.) to be relevant to the results shown.

The total and exoenzyme activities of the diploid strains are shown for whole cultures assayed by the Perret method, and for the diploid population in these cultures, calculated from the assayed values by correcting for the activities of the haploid segregants present in each culture.* The values of the haploid activities used in the calculations were those obtained for the relevant segregants; for the results, in parenthesis, the values for the parental strains were used.

Strain	Penicillinase activities of induced cultures (assayed values) ≠			Penicillinase activities corrected for the number of segregants in the assayed culture *≠		
	Total enzyme (U/mg.)	Exoenzyme (U/mg.)	Exoenzyme (% of total)	Total enzyme (U/mg.)	Exoenzyme (U/mg.)	Exoenzyme (% of total)
(a) Parental strain 147i (recipient)#	150	6·8	4·6			
Diploid (1)	57	4·3	7·5	48(42)	4·5(3·8)	9·3(9·0)
Segregant from diploid (1) 147i	105	4·1	3·9			
Diploid (2)	44	3·0	6·7	38(35)	2·8(2·8)	7·2(8·2)
Segregant from diploid (2) 147i	118	6·1	5·2			
(b) Parental strain 147C223 # (recipient)	369	15·4	4·2			
Diploid	127	10·4	8·2	121(126)	10·3(13·7)	8·5(10·9)
Segregant 147C223	412	21·3	5·2			

* The composition of the assayed cultures in (a) was from diploid (1) 80-87% diploid cells; 2-5% α-segregant, 8-18% β-segregant, and the proportions of each type from diploid (2) were respectively 86-90%, 2-6% and 8-11%; in (b) the proportions were 94-95%, 3% and 3-4% respectively.

≠ Unless otherwise stated the results are the mean of 3 values in each case.

\# Mean of 2 values in each case.

6. CONCLUSIONS

The penicillinase micro-constitutive mutant, P45, derived from the strain 8325: $\underline{a}\ \underline{i}^{+}\ \underline{p}_{A}^{+}$, makes small amounts of enzyme protein and has penicillinase activity less than that of the uninduced wild type strain. The restriction must be due to a mutation affecting enzyme synthesis, as the strain does not produce a factor destroying penicillinase, and this mutation was linked to the penicillinase structural gene in transduction experiments. The results of the experiments above (Section V. 2-5) with the primary mutant, and with revertants and recombinants of it, suggest that the genotype of P45 is $\underline{i}_{R}^{+}\ \underline{i}_{N}^{-}\ (\underline{o}^{+}\ \underline{pr}^{+})\ \underline{p}_{A}^{-}$. The reasoning by which this conclusion was reached is summarised below.

(a) The structural gene of P45

The mutant P45 has insufficient enzyme activity for it to be possible to decide unequivocally by serological tests whether this strain synthesises a mutant penicillinase molecule or not. Similarly, because of the difficulty in obtaining sufficiently high concentrations of the enzyme, satisfactory studies of thermolability were also not possible. However, the results of examination of the revertants to increased penicillinase activity and of recombination of P45 with an $\underline{i}_{R}^{-}$ allele suggest that the structural gene of P45 is $\underline{p}_{A}^{-}$.

(i) Reversion evidence

Revertants to increased penicillinase activity, induced in P45 by mutation with either NG or EMS, all had activities that

were less than the magno-constitutive level expected if P45 were $\underline{p}^+$, the maximum revertant level being about 20 - 25% of this value. In a representative sample of 11 NG-induced and 11 EMS-induced revertants, making a variety of levels of penicillinase activity, all the isolates were found to be making an enzyme protein that differed from normal A-type penicillinase and, hence, all must have an altered structural gene ($\underline{p}^-$). In every case, the enzyme was more temperature sensitive than the wild type enzyme. Penicillinase from the revertant N3 was also shown to be altered in its serological properties, and its relative activity is possibly about 20% of unmutated A-type enzyme. The change in enzyme structure of these revertants might be due to a pre-existing $\underline{p}^-$ mutation of the original mutant, P45, might be an essential part of the derestriction of penicillinase activity for which the revertants were isolated, or might be due to a second mutation at another site, which in this case is in the $\underline{p}$-gene. This last explanation is particularly possible for the revertants obtained by treatment with NG, because this has been found to cause multi-site mutations readily (Eisenstark, Eisenstark & Van Sickle, 1965). However, if this were the case, it would be unlikely for all the second-site mutations to cause a similar structural gene alteration, conferring temperature sensitivity on the enzyme, of mutants in which the other mutagenic changes are dissimilar, as evidenced by the differences in the total enzymic activity of these strains. It would also be unlikely to find a similar change in all the mutants induced with another mutagen,

EMS, since the two mutagens frequently act in different ways, NG producing mainly transitions and EMS transversions and deletions as well as transitions (Baker & Tessman, 1968; Eisenstark et al., 1965; Krieg, 1963; Tessman, Poddar & Kumar, 1964). If the $\underline{p}^-$ mutation were an essential part of the reversion process, a similar argument applies and a mutation to temperature sensitivity would not be expected to occur in all the revertants. Furthermore, in this case, if a mutation in $\underline{p}$ is required to remove the micro-phenotype then one implication is that the original micro-mutation also involved this gene. Therefore the universality of this enzymic property of the revertants is suggestive of a pre-existing mutation in the structural gene of P45, but does not establish this point. More definitive evidence comes from the recombinational experiments.

(ii) Recombinational evidence

When P45 was crossed with the strain P2C ($\underline{a}\ \underline{i}_R^-\ \underline{p}_2^-\ \underline{Em}^S$), less than $1/10^4$ of the erythromycin-resistant transductants were magno-constitutive recombinants, but recombinants with meso-constitutive phenotype occurred at a frequency of about 1/750. In similar experiments, Richmond (1966a) found magno-constitutive recombinants at 1/1,000 of the $\underline{Em}^R$ transductants from the cross $\underline{a}\ \underline{i}^+\ \underline{p}^+$ --- $\underline{\gamma}\ \underline{Em}^R$ x $\underline{a}\ \underline{i}_1^-\ \underline{p}_2^-\ \underline{Em}^S$, and at 1/5,000 - 1/7,000 of the $\underline{Em}^R$ transductants from the cross $\underline{a}\ \underline{i}^+$ micro $\underline{p}^+\ \underline{Em}^R$ x $\underline{a}\ \underline{i}_1^-\ \underline{p}_2^-\ \underline{Em}^S$ (using his micro-inducible and micro-constitutive strains), and the failure to find magno-constitutive recombinants in these experiments suggests, therefore, that P45 does not carry a $\underline{p}^+$ allele.

Furthermore, as the frequency of recombination to meso-constitutive is similar to that for a single cross-over between $\underline{i}_1^-$ and $\underline{p}_2^-$ (Richmond, 1966a), P45 does not appear to carry a synthesis-restricting gene between these regions. The meso-constitutive recombinants (rec 5 has been taken as an example) had higher penicillinase activity than the parental strain P2C, and made a protein that was clearly mutant on the basis of thermolability and reaction with antiserum. This protein could not be isolated by the usual method for purification of staphylococcal penicillinase. In all these properties the enzyme synthesised by rec 5 is distinguished from the A_2 mutant penicillinase made by the strain P2C which was used as the recipient in the cross. Therefore, the mutant enzyme made by rec 5 is not simply A_2 enzyme determined by the gene $\underline{p}_{A2}^-$ under the influence of a different control region. The recombinant, rec 5, may carry two point mutations in the structural gene, one derived from P45 and the other being the $\underline{p}_2^-$ mutation. If this were so, it would mean that recombination had occurred within the penicillinase gene, but this possibility is unlikely in view of the high frequency of recombination to the rec 5 phenotype. If peptide map analysis of rec 5 protein had been possible it would have proved this point definitively, but this analysis could not be carried out, because of the low yields of the rec 5 penicillinase eluted from cellulose phosphate and recovered after gel-filtration through Sephadex G-100. The active enzyme that was recovered was found to be heavily contaminated with another protein. All

attempts to improve the yield at this stage of elution from cellulose phosphate were unsuccessful. From the evidence available, it is concluded that rec 5 carries an altered penicillinase structural gene, and that this is the same gene that is carried by the parental strain P45, i.e., it is $\underline{p}^-_{45}$. From the serological experiments, the relative activity of the enzyme determined by this gene is possibly about 20% of the unmutated A-type penicillinase, and is similar to the enzyme made by the revertant N3.

(b) The regulator genes of P45

(i) The gene ($\underline{i}_R$) controlling the repressed level of penicillinase.

Despite its constitutive phenotype, strain P45 appears to carry an intact $\underline{i}^+$ gene as originally defined (Novick & Richmond, 1965) in that heterodiploids of the plasmid from P45 with the $\underline{\beta i}^-_{223}\ \underline{p}^+_C$ plasmid, carrying a magno-constitutive mutation of $\underline{i}$, have the repressed basal levels of enzyme characteristic of an $\underline{i}^+/\underline{i}^-$ diploid. The gene determining the uninduced level of penicillinase has been renamed above as $\underline{i}_R$ (see Section III.5.(f)) and trans-dominant repression of the expression of a structural gene linked with an $\underline{i}^-_R$ mutation, as occurs in these diploids, is taken as evidence of the presence of an $\underline{i}^+_R$ allele. Furthermore, if the enzyme synthesised by P45 has a relative activity of 1/5 of normal enzyme protein (as suggested above), then the basal activity, about 0·6 U/mg., of this strain is equivalent, in the amount of enzyme protein made, to about 3 U/mg. of unmutated enzyme,

which is the activity of the repressed wild-type strain.

Further evidence for the absence of an $\underline{i}_R^-$ mutation in P45 comes from the recombination studies. Firstly, in the transductional cross between the erythromycin resistant version of the plasmid from strain P45 and an $\underline{a}\ \underline{i}_R^+\ \underline{p}_A^+$ plasmid, no de-repressed recombinants ($\underline{i}_R^-\ \underline{p}^+$) were obtained in an experiment in which they would have been detected had they occurred at a frequency of more than 1/9,000 of the erythromycin-resistant transductants. The expected frequency of recombination between $\underline{i}_R$ and $\underline{p}$ is of the order of 1/1,000 of the erythromycin-resistant transductants (see Section V.6.(a)(ii) above) but as discussed in the Methods Section (Section II.11.(b)) strict comparison between different crosses may not be valid. Secondly, if a regulator constitutive ($\underline{i}_R^-$) allele is recombined with the structural gene from P45, as appears to be the case in rec 5, then the uninduced level of penicillinase expression is increased about 100-fold, which is similar to the value obtained on mutation of the wild type strain, 8325: $\underline{a}\ \underline{i}_R^+\ \underline{p}^+$, to its magno-constitutive derivative, 8325: $\underline{a}\ \underline{i}_R^-\ \underline{p}^+$. This factor of increase in the level of penicillinase expression would be less if P45 already carried an $\underline{i}_R^-$ mutation. (The regulator gene mutation ($\underline{i}_R^-$) of the parental strain P2C allows the magno-constitutive level of synthesis of penicillinase protein, and the presence of this allele in the recombinant rec 5 was confirmed by the dominant effect of an $\underline{i}_R^+$ allele in the *trans* position).

Reversion of P45 to increased enzyme levels, was associated with mutations in the repressor $\underline{i}_R$ region. These isolates were

either revertants to meso-levels of penicillinase activity, like N3, sensitive to repression by a *trans* $\underline{i}_R^+$ allele, or revertants to low levels of activity like N6, N46, N54 and N68, which had lost the ability to repress *trans* fully the enzyme determined by the $\underline{\beta}\ \underline{i}^-\ \underline{p}_C^+$ plasmid. The low activity of these latter revertants must have some other cause than repressor activity, perhaps a further mutation to the enzyme or a micro-mutation of the type described by Richmond (1966a & b). This evidence further supports the theory that P45, itself, is $\underline{i}_R^+$ where $\underline{i}_R$ is a regulator gene specifying the repressor function.

(ii) The gene ($\underline{i}_N$) controlling the induced level of penicillinase.

Although P45 has the repression properties associated with an $\underline{i}_R^+$ gene, it does not display the other basic property of inducible regulation, that of removal of repression (induction) in the presence of an inducer, thus suggesting a mutation in a region outside of $\underline{i}_R^+$. The results of transduction experiments show that this lesion is closely linked genetically to both the $\underline{p}$ and $\underline{i}_R$ genes carried on the same plasmid. When a diploid was formed between the plasmid from strain P45 and either the un-mutated plasmid $\underline{\beta}\ \underline{i}^+\ \underline{p}_C^+$, or the $\underline{\beta}\ \underline{i}^-\ \underline{p}_C^+$ plasmid of strain 147C223, induction of the structural gene $\underline{p}_{45}^-$ of strain P45 was restored. The evidence for this was that the proportion of exoenzyme released from the induced diploids was consistent with a fully induced $\underline{p}_{45}^-$ gene specifying a mutein of A-penicillinase with relative activity of 1/5 normal. From the diploids, this

proportion of extracellular enzyme was about 10% compared with about 5% from the haploid β-plasmid. From the α-plasmid alone, 30 - 50% of the penicillinase was extracellular. This method is necessarily only a rough guide to enzyme induction which was not always obvious in the results of individual experiments, as the release of cell-bound enzyme has a complex mechanism, dependent on such factors as polyanions and glucose in the medium as well as on the genetic constitution of the strain (Coles & Gross, 1967a & b). The total enzymic activity of these diploids making a mixture of normal and mutein penicillinases was less than that of a haploid strain making normal penicillinase. From the behaviour of these diploids, the mutation in P45 causing loss of inducibility is trans-recessive to a wild type allele on the β-plasmid, and, furthermore, in the diploid in strain 1470223, in which the repressed level of enzyme is determined by a gene (i_R^+) carried on the plasmid from P45, the region containing this mutation is complemented independently to the i_R region. This behaviour is similar to that of the other mutants of the induction response, the baso-constitutive and semi-inducible mutants studied by Richmond (1967b) (see Section III.4, above), and the meso-constitutive mutant K19 (Section III.5.), on the basis of which a second regulatory locus (i_N) has been proposed. Therefore it is concluded that P45 also has a mutation in this region (i_N^-). This loss of an induction function is also shared by all the revertants of P45 that were isolated, even under conditions specifically directed to detecting reversions of this property, though, of course, a gene specifying

inducibility would not be detectable in a fully derepressed mutant.

(c) The operator and promoter genes of P45

(i) The operator region

The characteristics of the micro-constitutive mutant of penicillinase production, P45, can be explained on the basis of a structural gene mutation and of the same, or a second, mutation affecting a regulatory region (i_N) concerned with an induction function. The gene (i_R^+) determining the repressed level of enzyme appears to be fully functional, suggesting that a region analogous to the operator region of the lac system in E. coli is unimpaired (o^+) in this mutant. Consistent with the conclusion that P45 is not operator constitutive (o^c) is the failure to find high level constitutives among the progeny from the cross with $a\ i^+\ p^+$, though if such a recombination is a rare event it may not have been found in the number of transductants screened (9,300). Furthermore, the behaviour of the diploids formed with an i_R^+ allele in the trans position to either the mutant N3 or the recombinant rec 5 suggests that the expression of the p_{45}^- gene is still linked to a functional operator region (o^+) in N3 and rec 5, although the repression of the P45 mutant has been removed by replacement of the i_R^+ by an i_R^- allele.

(ii) The promoter region

In complementing diploids, repression could be removed from P45 penicillinase synthesis, and the activity expressed was 1/5 the normal amount, which is consistent with the view that the amount of

protein made was similar to the normal maximum but had 1/5 of the wild type activity, a characteristic of $\underline{p}^-_{45}$. Hence there appears to be no restriction on penicillinase synthesis in P45 of the kind expected of a promoter ($\underline{pr}^-$), an initiator (Margolin & Bauerle, 1966), or an operator-zero ($\underline{o}^o$) mutant. When repression of penicillinase synthesis was removed by mutation of the $\underline{i}^+_R$ to $\underline{i}^-_R$, a number of the strains produced had the enzyme levels expected for full expression of the $\underline{p}^-_{45}$ mutant enzyme, so that if the original mutant, P45, were $\underline{pr}^-$, then reversion of this mutation occurs at high frequency (about 10^{-4}). Similarly there is no evidence of $\underline{pr}^-$ linked to $\underline{p}^-_{45}$ in the recombinant rec 5. In this case it is possible that such a mutation carried by P45 has been replaced by the wild type allele from the strain P2C but the high frequency of recombination to the rec 5 phenotype discredits this idea, if the promoter region is closely linked to the structural gene. For this view to be tenable, the mutation would have to lie in a region located at the start of another cistron co-regulated with penicillinase, as is the case for the later cistrons of the lac operon in E. coli (Jacob et al., 1964) and some genes of the trp operon of Salmonella (Margolin & Bauerle, 1966).

In summary, the behaviour of P45 is consistent with the genetic composition $\underline{a}$ $\underline{i}^+_R$ (repression) $\underline{i}^-_N$ (induction) $\underline{o}^+$ $\underline{pr}^+$ (promoter) $\underline{p}^-_{45}$. P45 shows no evidence of having a 'micro' mutation like that of the micro-constitutives examined elsewhere (Richmond, 1966a & b) which is cis-dominant, is outside the p-gene, and which is a point mutation revertible to give an inducible

phenotype. The mutational events which might give rise to the P45 genotype are discussed in the general Discussion (Section VI.4, below).

(d) The revertant N3 of strain P45

Some aspects of the behaviour of the revertant N3 are of interest. This strain was isolated as one of a group of NG-induced mutations of strain P45, and similar strains do not readily arise by spontaneous mutation as shown by its absence amongst 10^4 transductants of the plasmid-negative strain 8325N with the plasmid of strain P45, in an experiment designed to detect spontaneous revertants and transductional artefacts. N3 is a derepressed strain and behaves like a plasmid linked $\underline{i}_R^-$ mutant. The constitutive phenotype was retained on transduction to the plasmid-less host strain 147 N, and was repressed in a diploid formed with the unmutated plasmid $\underline{\beta}\ \underline{i}^+\ \underline{p}_C^+$. However, diploid clones formed with strain 147C223 (147: $\underline{\beta}\ \underline{i}_{223}^-\ \underline{p}_C^+$), segregated again rapidly. Unlike the 'unstable' diploids due to a host mutation (Richmond, 1967a) both plasmids were not lost together, but one or other parental type was retained. This behaviour resembles, rather, other plasmid and host strain mutations affecting plasmid maintenance (Novick, 1967a & b). Similar 'unstable' (rapidly segregating) diploids were formed in strain 147C223 by the plasmids from the other revertants of P45 which were tested, and which behaved in these diploids in the manner expected of mutants of the $\underline{i}_R^-$ type. Diploids with the parental plasmid (P45) in this strain were relatively stable, and with the wild type $\underline{\alpha}\ \underline{i}_R^+\ \underline{p}_A^+$-

plasmid highly so. The plasmid carrying the meso-constitutive mutation of K19 had also formed stable diploids. The origin and nature of the instability of these diploids, and any relevance to it of some i_R^- mutations, is not clear.

As a rare event with the plasmid from N3 a more stable diploid, with unusual properties, was formed. This, unexpectedly for a diploid of two regulator i_R^- (derepressed) parent alleles, had the basal enzyme levels of a repressed diploid. Haploid segregants from the first such diploid apparently retained the parental penicillinase phenotypes, but the α-segregant (originally N3) from later diploids had apparently reverted spontaneously to low levels of penicillinase synthesis.

Another difference between the two groups of segregant was that, in the first case, the segregants were isolated before storage of the diploids in the cold room, in the second case they were isolated within 3 days of the time of assaying the diploids, two months after the initial isolation. Segregants of the first diploid, obtained at this later stage, were not assayed by the Perret method, but were rapidly screened by the penicillin-iodine reagent on growth on CY agar. This test is not always accurate, especially if there are many strains being tested on each plate or the enzyme activities are widely varied. Furthermore, the activity of the control N3 strain appeared very small on this test, perhaps because of the thermo-lability of the enzyme, so that the α-segregants from the later diploids were not clearly distinguished from N3 by this method. It is therefore possible that the N3-

plasmid in the first diploid had, by the time the diploid was assayed also undergone genetic modification affecting the basal level of enzyme. Unfortunately, this strain was no longer available for test.

If, as the initial results indicated, both plasmids in the diploid carry their original $\underline{i}_R^-$ alleles, then an explanation of restoration of repressor activity would include the possibility of interallelic complementation, implying that the functional repressor is polymeric. However, this observation is suspect and was not repeatable; instead a change was found in the phenotype determined by the originally N3 $\underline{\alpha}$-plasmid. The nature of this change was not established conclusively, but the replacement of N3 levels by low levels of enzyme, and the failure of a magno-constitutive phenotype to be expressed in diploids with it, suggest a reversion to $\underline{i}_R^+$, i.e. restoration of repressor activity. This might have arisen by recombination between the two $\underline{i}^-$ mutants since another unusual feature of the first diploid isolate was the recombination between the $\underline{\alpha}$- and $\underline{\beta}$-plasmids for the other linked markers, $\underline{Cd}^R$ and $\underline{Em}^R$. Such recombination is a rare event in normal $\underline{\alpha}/\underline{\beta}$ diploids but does occur in some 'unstable' diploids (Richmond, 1967a). Alternatively, the mutation giving rise to the strain N3 may be an unstable mutation. The original haploid strain N3 has not been examined for the spontaneous reversion rate. Examples of unstable suppressor mutations of this postulated kind have been reported by Dawson & Smith-Keary (1963), Morse (1967), Riyasaty & Dawson (1967) and Schwartz (1967a & b).

The problems raised in these experiments were not pursued further at this stage as they were peripheral to the understanding of the micro-mutant P45.

VI. DISCUSSION

There are several different classes of mutant of *S. aureus* in which penicillinase is synthesised constitutively. Examples of some of these classes have been produced, and the regulation of the enzyme and the genetic constitution of representative mutants studied. In the following sections the results are discussed for the different categories of mutants separately, followed by a general discussion of the whole system in the light of the findings.

1. OPERATOR-CONSTITUTIVE MUTANTS

Mutants of the operator region, with the complementation characteristics of operator-constitutive (O^c) mutants, as isolated for the *lac* operon of *E. coli* (Jacob & Monod, 1961; Jacob *et al.*, 1964), were sought as evidence of an operator region for staphylococcal penicillinase. The characteristics expected for mutants of this type are *cis*-dominant expression of derepressed penicillinase synthesis. No such mutants have been found among the haploid mutants of penicillinase that have been examined, and neither, in an experiment specially designed to facilitate their isolation, were any found amongst the mutants of a strain diploid for the penicillinase regions. Therefore there is, at present, no direct evidence for an operator (*O*) region for staphylococcal penicillinase. However, the failure to isolate O^c mutants does not necessarily mean that *O* does not exist in this system. The

mutation rate to $\underline{o}^c$ might be lower than the rates detectable in these experiments (10^{-4} - 10^{-5} with EMS, and 10^{-5} with X-rays as the mutagen), or might be obscured by a concurrent mutation at another site. Mutation to $\underline{i}^-$ occurred at a frequency of about 10^{-3}, and mutations leading to partial or total loss of a plasmid were readily found within the scope of the experiments. A search among a greater number of mutants might yet reveal one of the $\underline{o}^c$ type. Its isolation might be facilitated further by the use of a diploid strain carrying the plasmid markers $\underline{\alpha}$ $\underline{Cd}^S$ $\underline{Em}^R/\underline{\beta}$ $\underline{Cd}^R$ $\underline{Em}^S$ so that selection against mutations leading to the loss of a whole plasmid is possible by inclusion of both erythromycin and cadmium ions in the agar medium on which colonies are grown in order to screen for mutations.

The magno-constitutive mutation reported by Cohen & Sweeney (1967) is distinct from those of the regulator gene (i), and has not been tested in heterodiploids for its dominance or recessiveness. However, this mutation is not linked to the plasmid-borne penicillinase gene (p), and so could not, by definition, be in an operator region.

The only cis-dominant constitutive mutants that have been found for penicillinase production of S. aureus and that might be ascribed to a mutation of an operator region, are the micro-constitutive mutants described by Richmond (1966a & b). However, mutants in which the level of expression of an operon is limited and shows no increase after induction have been isolated in other systems, and have been shown to be caused by mutations located

outside of O, for example, the lac_2 mutation (Beckwith, 1964a; Brenner & Beckwith, 1965) and gal_3 (Hill & Echols, 1966) in the lac and gal operons, respectively, of E. coli. The micro-constitutive mutants described by Richmond might be polar mutants similar to one of these types.

2. MAGNO-CONSTITUTIVE AND SEMI-CONSTITUTIVE MUTANTS

Penicillinase is an inducible enzyme in S. aureus. A gene (i) concerned in the regulation of the rate of penicillinase expression was originally identified by Novick & Richmond (1965). The state of this gene controls the basal level of enzyme, and it was recognised by mutations (i^-) of it, the magno- and semi-constitutive mutations resulting in an increase in the amount of penicillinase made in the absence of an inducer. The mutant form of this gene (i^-) is trans-recessive in diploids to the unmutated i^+. Regulatory mutants, which behave in this way, can be interpreted as having a cytoplasmic product of the i-gene, the repressor, which, in its unmutated form, is active in preventing the expression of a particular gene, but which is altered by interaction with a specific inducer molecule so as to permit synthesis of the regulated enzyme. An i^- allele either does not make this product, or makes one which does not interfere with the activity of the i^+ gene product acting trans in the same cell. Mutants of the semi-constitutive phenotype indicate that an altered product is possible, one that is impaired in its activity as a repressor but is not completely inactive, and will still interact with an inducer molecule

to allow enzyme induction. The cytoplasmic product determined by the *i*-gene, with the characteristics of a repressor, may be a protein which interacts with the operator region next to the regulated genes, probably on the DNA thus preventing messenger RNA transcription, but possibly on the RNA to prevent translation, as is thought to be the case for the *lac* operon of *E. coli* (Beckwith, 1967); or the product may be the messenger RNA itself on which the *i*-gene is co-transcribed with the regulated genes and exerts its repression effect at the level of translation, as suggested above (Section III.2). The second hypothesis is less easily reconciled with the current theories of protein synthesis, reviewed by Lengyel (1966), and it does not readily present a mechanism at the molecular level for the specificity of induction. However, for the penicillinase system there is at present no evidence which distinguishes between these two possibilities.

Since the results of the meso-constitutive mutant indicate that a second locus is involved in the inducible regulation of penicillinase, and this is consistent with the conclusion reached by Richmond (1967b) for baso-constitutive and semi-inducible mutants, the regulator gene (*i*) determining the repressed level of enzyme synthesis, and mutated to i^- in magno- and semi-constitutive strains, is referred to as i_R to distinguish it from the second locus, i_N.

3. MESO-CONSTITUTIVE, BASO-CONSTITUTIVE and SEMI-INDUCIBLE MUTANTS

Mutants with the meso- and baso-constitutive and semi-inducible phenotypes are all impaired in the level of enzyme attainable in the presence of inducers. A meso-constitutive mutant, K19, was examined and, in this type of mutant, as well as the loss of inducibility, the activity of the repressor was partially impaired. From the complementation data, the conclusion was reached that two separate cistrons are involved in the inducible regulation of penicillinase, one concerned with the repression function and the other with induction. The same conclusion has also been drawn from the results of complementation experiments with baso-constitutive and semi-inducible mutants (Richmond, 1967b). The two cistrons distinguished in this way, $\underline{i}_R$ controlling the repressed enzyme level and $\underline{i}_N$ the induced level, can behave as independent regions mutationally. In magno- and semi-constitutive mutants only $\underline{i}_R$ is altered, and in baso-constitutive and semi-inducible mutants only $\underline{i}_N$. Both activities may be completely abolished, as in the magno-constitutive and baso-constitutive mutants respectively, or may only be partially lost with a proportion of the original activity remaining, as in semi-constitutive and semi-inducible mutants. The mutant forms of both genes, $\underline{i}_R^-$ and $\underline{i}_N^-$, are trans-recessive to the wild type $\underline{i}_R^+$ and $\underline{i}_N^+$.

Study of the mutant K19 showed that the $\underline{i}_N$ gene does not correspond to a promoter region (Jacob et al., 1964) but that it has some other regulatory function. The function of the product

determined by this gene might be attributed to (a) an enzyme converting the inducer (I) into the true inducer (I'); (b) a permease transporting the inducer to its site of function; (c) an element for positive control of the penicillinase gene; (d) a part of the repressor molecule containing the site of interaction with the inducer.

(a) An enzyme for inducer conversion

The i_N control region could be the determinant for an enzyme which converts the added inducer, I, into the true inducer, I', for penicillinase induction. Both methicillin and cephalosporin C would be substrates of such an enzyme, and in the non-inducible mutant K19 the specificities for both of these have been lost. If there were any residual activity left in the mutant form of such an enzyme then some of the product, I', might be expected to be formed, particularly when the concentration of I is increased. No evidence of induction was found with a range of concentrations of either inducer, but the increase above the optimum amount for induction of the wild type strain, P_o, was limited by the concentration at which bacteriostasis interfered with the assay. If inducibility is dependent on such an enzyme, and the loss of inducibility is the result of mutation of its structure, then a very drastic alteration amounting to complete loss of all enzymic activity would have to be postulated. In other mutants in which the induction response can be shown to be altered, the baso-constitutive and the semi-inducible mutants, examined by Richmond (1967b), the same result of complete failure to force induction by

increasing the inducer concentration also applied. The behaviour of semi-inducible mutants, in particular, argues against the hypothesis of an enzyme converting the inducer into its active form. These mutants retain some induction response, therefore the enzyme must still have some activity for the substrate, though at a reduced rate. In such a case, increase in the substrate concentration might be expected to lead to an increase in the product concentration which, in this case, would be reflected in an increase in the induced level of penicillinase.

(b) A permease

Another possible hypothesis is that a permease is required to transport the inducer molecule to its site of action across some barrier such as the cell membrane. Basically similar arguments to the previous ones, used for the enzyme suggested above, apply to the failure to force the barrier by increasing the concentration of inducers, and, again, the limitation to the increase of concentration, caused by antibacterial activity, may have meant that sufficiently high concentrations of inducer had not been reached. These two possible explanations, of a converting enzyme or a permease, cannot be excluded, though, for both, the fact that two substrate specificities have been lost simultaneously must mean that gross alteration has occurred, affecting several amino acids (by, say, deletion) or one functionally important amino acid.

(c) An element for positive control

Synthesis of penicillinase may depend on the presence, in an active form, of a regulator molecule, which may be, but need not

necessarily be, another form of the repressor molecule. This type of positive control of an operon has been proposed for the l-arabinose system of E. coli (Sheppard & Englesberg, 1966) on the basis of a magno-constitutive mutation which is trans-dominant to the wild type allele of an inducible strain. These authors suggest that the active form of this regulator substance occurs in the presence of the inducer and is permanently present in their constitutive mutant resulting in full expression of the operon. Loss of operon expression follows an inactivating change in the controlling molecule. The non-inducible mutants of penicillinase in S. aureus might be due to mutation affecting an element of positive control similar to that for the l-ara system. However, no evidence of this form of control has been found for penicillinase. All derepressed mutants are recessive in heterodiploids and unlike the l-ara constitutive mutant. In preliminary experiments, a mutant of S. aureus non-inducible for penicillinase, the baso-constitutive strain P47, was mutated to levels of enzyme synthesis similar to meso- and magno-constitutive strains, without restoration of inducibility to any of the mutants in which this was testable. Therefore, the level of enzyme synthesis does not require the product of an unmutated (active) form of the i_N gene, and the locus for "induction response" behaves, mutationally at least, as an entity distinct from a locus necessary for the production of penicillinase.

(d) The repressor molecule

Another possible explanation of the loss of inducibility is

that part of the repressor molecule itself has been affected. This explanation has been put forward for the i^s (super-repressed) regulatory mutants for the synthesis of β-galactosidase in *E. coli* (Willson *et al.*, 1964). These mutants map in the *i*-gene of this system and appear to be due to changes in the site on the repressor molecule for the recognition of the inducer such that the specificity for the inducer has been altered or completely lost (Bourgeois, Ph.D. Thesis). The i^s phenotype is dominant over the i^+ in heterogenotes. In the mutant, K19, of the penicillinase system of *S. aureus*, if such a change of specificity for the inducer has occurred, it must be sufficiently gross to exclude reaction with two compounds which are powerful inducers of the wild type strain. However, in contrast to the *lac* system, the property, non-inducibility, is *trans*-recessive to inducibility, and in heterodiploid strains complementation of this property occurs independently of repressor activity, suggesting that two separate cistrons are involved, one specifying each of the two properties, repression and induction.

If both these properties belong to the cytoplasmic repressor itself, then the occurrence of mutual complementation indicates that the repressor is composed of more than one subunit. This polymer might contain identical subunits or different ones, in the first case both i_R and i_N being translated into a single, continuous polypeptide, and in the second case into two separate ones. Since the extent of mutual complementation between the two characteristics specified by these genes is nearly complete for all the mutants

tested, it seems more likely that the polymer is composed of two different subunits carrying separately the active centres for repression of protein synthesis and for inducer interaction to remove this repression. It should be noted, however, that it becomes increasingly difficult to predict the complementation properties of heterogenotes when the number of subunits in the polymer is increased. A necessary part of the polymeric hypothesis is that the two types of subunit must be able to combine with each other, and a class of mutant, similar to the lac i^s mutants in behaviour, can be postulated in which the repressor subunit, while still able to repress, has lost this ability to combine with the subunit containing the inducer site. A similar loss on the "inducer" subunit would appear identical to a non-inducible mutant, such as K19, which is trans-recessive in heterogenotes with the wild type allele, and this is another possible explanation of the staphylococcal mutants non-inducible for penicillinase.

(e) The location of the i_N gene

The two regulatory genes postulated, i_R and i_N, may be mutated independently, but in the meso-constitutive mutants both appear to be altered. The frequency of occurrence of mutants with a meso-constitutive phenotype is about the same as for magno- or semi-constitutive mutants (see Section III.1) believed to be due to single-site mutations, suggesting that the events producing a meso-constitutive phenotype are also single site mutations. If this type of mutant were due to two separate mutations, one in each

cistron, recombination between them should be possible. In an attempt to recombine between two such mutations in K19, no recombinants of this region were found in over 10^5 transductants receiving the erythromycin resistance marker. Richmond (1966a) found recombination rates of $1/10^3$ among the erythromycin resistant transductants in the region between an $\underline{i}_R^-$ mutation and a structural gene mutation, $\underline{p}_2^-$, and of greater than $1/10^4$ among the erythromycin resistant transductants in the region between $\underline{p}_2^-$ and a determinant for the micro-phenotype, thought to be next to the structural gene, $\underline{p}$. Thus, if K19 has two point mutations, they are so close together that recombination between them is an extremely infrequent event, but more probably it is a single site mutation. To confirm this conclusion, it would be of interest to show recombination of the type attempted with K19 with a meso-constitutive strain constructed in two independent mutational steps, and to compare the rate of recombination between two separate mutant loci with that obtained for K19.

From the evidence presented, the meso-constitutive strain, K19, probably arose from a single site mutation, but two functions, apparently in two cistrons, have been affected. This result could follow from a polarity mutation, such as a frame-shift mutation, or from an extensive deletion affecting two neighbouring cistrons. The genetic map positions of these two cistrons with respect to the structural gene for the enzyme are not known.

4. MICRO-CONSTITUTIVE MUTANTS

The micro-constitutive mutant P45 is not inducible by more than a factor of two, and has penicillinase activities of less than the uninduced wild type strain. Examination of this mutant leads to the conclusion that its properties are due to the genotype $\underline{\alpha}\ \underline{i}_R^+\ \underline{i}_N^-\ \underline{p}_{A45}^-$. There was no evidence of promoter (or initiator) or operator mutations. The possible derivations of this mutant genotype are discussed below as regards the nature and relationship of the genetic regions involved.

P45, a mutant in two properties, enzyme structure and response to inducer, was derived from the wild-type $\underline{\alpha}\ \underline{i}^+\ \underline{p}_A^+$ by one mutational treatment with EMS. The resulting mutation may be attributable to one event at one chromosomal site or by two events at two separate sites. If it is one event then this may be (a) a non-polar point mutation, (b) a polarity mutation, or (c) a more extensive deletion.

(a) A non-polar point mutation

In the case that the original mutation was a single non-polar mutation, the same gene must affect both of the functions altered in P45. The evidence is against this hypothesis. Firstly, the induction of P45 in diploids is consistent with full complementation of the $\underline{i}_N^-$ region while the $\underline{p}^-$ region still produced altered protein of 1/5 activity. Thus complete and independent complementation of $\underline{i}_N^-$ occurred, the behaviour expected of a separate cistron. Secondly, although it is not possible to tell, in the absence of a $\underline{\beta}\ \underline{i}_N^-$ test strain, whether a recombinant of the $\underline{p}_{45}^-$

gene with a magno-constitutive $\underline{i}_R^-$ gene, as in rec 5, has also received an $\underline{i}_N^+$ allele or not, the observation was made that full induction of the mutant enzyme also occurred in complementing diploids with rec 5. The same observations apply to the revertant N3. So the functional separation of the two properties is maintained in derivatives of P45. Thirdly, no reversions to wild-type occurred; instead all revertants were of a suppressor type, that is to say, a further mutation at another site. True reversion of an EMS-induced mutation may be produced after EMS treatment (Krieg, 1963) but is less likely from NG treatment (Baker & Tessman, 1968), and as the suppressor mutation rate is high, $1/10^3$, true revertants might have been missed if occurring at low frequency, so this point is not conclusively proved.

(b) A polarity mutation

If the original mutational event was a single site polarity mutation the two properties $\underline{p}_{45}^-$ and $\underline{i}_N^-$ may be coded for by separate cistrons but these would be within the same operon, transcribed as one unit and translated sequentially. If this is so, then in this case $\underline{p}$ and $\underline{i}_N$ could well be adjacent cistrons; if not, the genes between them code for unknown properties which have not yet been detected. As the structure of the penicillinase molecule is altered but 20% of the original activity still remains the mutation causing polarity might well occur in this gene, the carboxyl terminus of the peptide chain being altered, and $\underline{i}_N$ being transcribed later than $\underline{p}$. This would suggest map order (i) in Fig. 8. The question then is what is the role of the product of

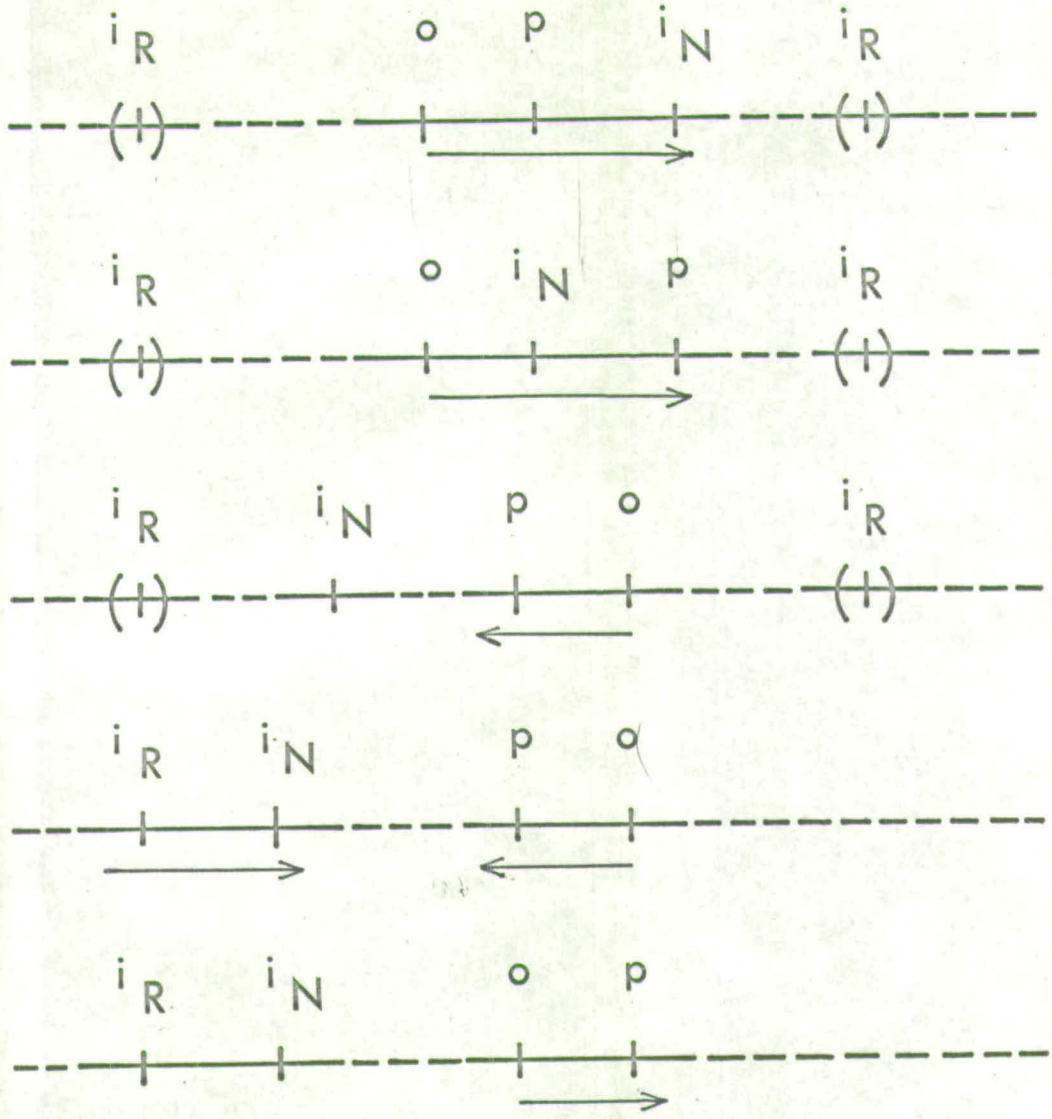


Fig. 8. Relative genetic locations possible for the regions concerned in the synthesis of penicillinase.

p = structural gene for penicillinase.

o = an operater region which may exist for p.

i_R = a regulator gene determining the repressed level of penicillinase synthesis.

i_N = a regulator gene determining the induced level of penicillinase synthesis.

← → = direction of genetic transcription.

i_R = alternative positions for i_R, either to the right or the left of p.

gene $\underline{i}_N$. Trans complementation suggests that it specifies a cytoplasmic product. As discussed above (Section VI.3) this product may form part of a normal polymeric repressor molecule, or it may have a function indirectly affecting induction of the repressor such as that of an enzyme converting the inducer molecule, I, into its active form, I', or of a permease making the inducer available to the repressor. If it forms part of the repressor, then expression of $\underline{i}_N$ as a regulator gene should be independent of p, the regulated gene, and not transcribed in the same messenger-RNA,(Fig. 8 (iii)-(v)), but with the other possibilities co-transcription is permissible,(Fig. 8 (i)-(ii)). The failure to find independent reversions to either $\underline{i}_N^+$ or $\underline{p}^+$ with EMS mutation does not favour the polarity hypothesis, as it is possible for a further mutation between the original mutational event and the gene suffering the polarity restriction to restore the function to the second gene.

(c) An extensive deletion

If the P45 mutation is an extensive deletion cutting into 2 cistrons, the same argument as for (b) applies to the proximity of these genes, but $\underline{i}_N$ may lie on either side of p with respect to the operator. The possibilities are shown in Fig. 8 as maps (i)-(iv). Map order (v) is eliminated by the fact that both operator and promoter for p are intact, and in this order they would be covered by the deletion. Maps (i) and (ii) imply that $\underline{i}_N$ is regulated with p; in (iii) and (iv) $\underline{i}_N$ is expressed independently of the p operon as would be expected of a regulator gene. From the

study of K19, evidence was obtained for the map proximity of the two regulator regions $\underline{i}_R$ (affecting repression) and $\underline{i}_N$ (affecting induction). If the $\underline{i}_N$ allele in P45 is the same as that in K19 then $\underline{i}_R$ and $\underline{i}_N$ must both lie on the same side of $\underline{p}$ and that is the side distal to the $\underline{p}$ operator. Furthermore, $\underline{i}_N$ must be nearer to $\underline{p}$ than is $\underline{i}_R$ to allow for both of these mutants, one with the $\underline{p}$-gene and its expression unchanged, the other with normal $\underline{i}_R^+$ repression function. The identity of the $\underline{i}_N$ alleles in the two mutants is inferred from the functional similarities of the mutants and their complementation behaviour, but is not proven. To prove this, data is needed for recombination between the different mutants and extensive complementation tests against $\underline{i}_N$ mutants of the $\underline{\beta}$-plasmid. The failure of either property of P45 to revert to normal is consistent with the original mutation being due to a deletion.

Little can be deduced of gene order in the case where P45 carries a multi-site mutation. This possibility should be distinguishable from a single site mutation by recombination between the two sites. Evidence to test this possibility would be found from the cross between the plasmid P45 ($\underline{a}\ \underline{i}_R^+\ \underline{i}_N^-\ \underline{p}_{45}^-$) with the plasmid of strain P2i, making a fully inducible enzyme determined by the $\underline{p}_2^-$ structural gene mutation ($\underline{a}\ \underline{i}_R^+\ \underline{i}_N^+\ \underline{p}_2^-$), where one such recombinant would be $\underline{i}_N^+\ \underline{p}_{45}^-$ and detectable among the other progeny of the transduction by its penicillinase activity in the presence of inducer. This isolation would be analogous to the identification (in the absence of inducer) of the de-repressed recombinant rec 5 from the cross of P45 x P2C.

If rec 5 enzyme, which is apparently determined by the P45 structural gene, could be successfully purified, then the actual amino acid changes and their position in the peptide could be determined. This would show whether it is a point mutant or a partial deletion, whether extending beyond the peptide, and, if so, over which end of the polypeptide, amino or carboxyl.

Fine mapping of the penicillinase structural and control regions in S. aureus would greatly help our understanding of the number and nature of the cistrons involved and in interpreting new mutants. However, so far mapping by 3 factor crosses of the few markers known for the penicillinase plasmid have given conflicting map orders for them (M.H. Richmond and R. Irvine, unpublished experiments). Until this problem has been resolved attempted maps are not meaningful.

5. GENERAL CONCLUSIONS

The inducible regulation of the enzyme, penicillinase, determined by the gene p, appears to be controlled by two genetic regions. One of these genes, i_R, is concerned with the determination of the uninduced level of synthesis of penicillinase, and the other, i_N, with the level of enzyme achieved in the presence of inducing compounds. These two genes are independently mutable, and are independently complemented in all the mutants of the α-plasmid for which this property has been tested in hetero-diploids with the β-plasmid carrying the magno-constitutive

mutation i^-_{R223}. It is, of course, possible that modifications of this conclusion might follow from complementation studies with other mutations of the β-plasmid.

In wild type inducible strains, i^+_R determines a repressed level of penicillinase activity of about 5 U/mg., and i^+_N inducibility to levels of about 300 U/mg. in the presence of an effector compound. Different mutations in either region cause alterations in phenotype which may be of complete loss of the function determined by the wild type allele, or may be of a partial loss of this activity. The extent of the impairment of function may have any value in the range between no activity and the full wild type activity.

Both the alleles, i^+_R and i^+_N, are *trans*-dominant over their mutant forms, i^-_R and i^-_N, implying that the function of both is by means of a cytoplasmic product specified by the gene concerned. For i_R, this may be a repressor molecule of a type similar to the repressor described for the *lac* operon of *E. coli*. The product of the gene, i_N, may be an enzyme involved in activating the inducer compound as added to the bacterial cells, or in transporting this compound to its active site, or it may be a subunit of the repressor molecule itself. No evidence was found for a function involving positive-control, in which case an active form of this product would be essential for enzyme expression.

Mutants phenotypically magno-constitutive and semi-constitutive are i^-_R i^+_N, and phenotypically baso-constitutive and semi-inducible are i^+_R i^-_N as was also the micro-constitutive mutant examined, in

this case the micro-phenotype being due to a $\underline{p}^-$ mutation. Meso-constitutive mutants are both $\underline{i}_R^-$ and $\underline{i}_N^-$.

These genes are located on the same plasmid as carries the structural gene, p, and the only information on their relative positions comes from the meso-constitutive mutant, K19, examined here. In this example, the mutations to $\underline{i}_R^-$ and $\underline{i}_N^-$ seem to be ascribable to a single mutational event. Information on the location of $\underline{i}_N$ in relation to p might be obtained from further recombinational studies with the micro-constitutive mutant P45 which is mutated in both these loci.

No evidence for an operator region was found in the work described above, but this negative evidence is insufficient to exclude the existence of such a region. Neither have any mutations of a promoter or initiator region been found among the mutants examined. However, such a region may exist for staphylococcal penicillinase, as the mutants of micro-phenotype, examined by Richmond (1966a & b), may be ascribable to alterations of a region with this type of function.

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